Presence of the protruding oncus is affected by developmental stage and acetolysis technique.

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Abstract:

A protruding oncus is a projection of the intine in the aperture region. The ubiquitous use of acetolysis in palynological research has led to the presence of a protruding oncus being underreported. Controlled experiments with pollen samples collected from undehisced and dehisced anthers demonstrate that the presence of a protruding oncus is affected by the state of the anther at maturity: dehisced or undehisced and by the preparation technique. In investigating the occurrence of onci, particular attention should be paid both to the dehiscence state of the anthers and the effect of the preparation technique on the intine. Although it has been suggested that protruding onci and pollen buds can be distinguished based on three criteria (size, presence of a large vacuole, separation of the protrusion from the grain), most of these distinctions break down when information is included from more recent studies. Additional study of protruding intinous structures may help clarifing the difference between pollen buds and protruding onci.

Keywords: pollen grain | intine | acetolysis | anther | oncus | pollen bud | Rubiaceae | biology | botany | plant evolution

Article:

The oncus (pl. onci) is an intinous structure occurring beneath the apertures of many types of pollen grains (Hyde, 1955). Previous studies have investigated the formation and function of the oncus during pollen development and germination (e.g. Heslop-Harrison & Heslop-Harrison, 1980; Barnes & Blackmore, 1986; Heslop-Harrison et al., 1986; Hansson & El-Ghazaly, 2000; Ben Saad-Limam et al., 2005), and there has been much discussion on the term and in relationship to its counterpart 'Zwischenkörper' (e.g. Hyde, 1955; Seoane-Camba & Suárez-Cervera, 1986; Rodríguez-García & Fernández, 1988; Ben Nasri-Ayachi & Nabli, 1995; Banks, 2003; Ben Saad-Limam et al., 2005). However, there has been little work on protruding onci, which have been reported to occur in the Rubiaceae (Tilney & Van Wyk, 1997; Hansson & El-

Ghazaly, 2000; Dessein et al., 2005; Ma et al., 2005; Vinckier & Smets, 2005; Kuang et al., 2008).

The term 'protruding oncus' was proposed by Tilney and Van Wyk (1997) to refer to intine protrusions (sometimes with protoplasts) that project through the pollen grain aperture. The term was proposed to replace 'pollen bud' following Weber and Igersheim's (1994) objection that 'pollen bud' was an inappropriate term. However, pollen buds have usually been described as containing large vacuoles (Igersheim & Weber, 1993; Hansson & El-Ghazaly, 2000; Dessein et al., 2005), whereas depictions of protruding onci show them lacking vacuoles or, at most, possessing very small vacuolar remnants (Tilney & Van Wyk, 1997; Kuang et al., 2008). This distinction was not made by Tilney and Van Wyk (1997). Pollen buds are also usually larger than protruding onci and appear to be shed from the pollen grain before anther dehiscence (Philip & Mathew, 1975; Chennaveeraiah & Shivakumar, 1983; Priyadarshan & Ramachandran, 1984; Igersheim & Weber, 1993; Weber & Igersheim, 1994), a feature that has not been observed for protruding onci. Thus, it seems prudent to follow Hansson and El-Ghazaly (2000) in distinguishing these two terms based on the size of the protrusion, the presence or absence of a well-developed vacuole and whether or not the protrusion separates from the grain before the grain is shed. In this paper, the term 'protruding oncus' will be used for smaller apertural intine protrusions with or without cytoplasmic inclusions, but which lack the large vacuoles that have been reported to occur in pollen buds. We return to an examination of the differences between protruding onci and pollen buds in the Discussion section.

As intinous structures, onci are not resistant to acetolysis (Hesse & Waha, 1989; Punt et al., 2007) and, as this paper demonstrates, neither are protruding onci. Unfortunately, most palynological research has been carried out using acetolysis, which destroys any protruding onci. Structures that are most likely protruding onci have been found in many Chinese species of Rubiaceae (Kuang et al., 2008; Kuang, unpublished data, 2011).

In this paper, we draw attention to the presence of protruding onci in the Rubiaceae and other angiosperms, investigate factors that may have caused the presence of protruding onci to be underreported and discuss the distinctions between pollen buds and protruding onci based on our current knowledge of these structures.

Material and methods

Unopened flowers of Uncaria hirsuta Havil. with polliniferous anthers were collected from living plants growing in the South China Botanical Garden, Guangzhou, Guangdong Province, China. Uncaria hirsuta was chosen for study because of its possession of pollen with obvious protruding onci and because sufficient living material was available for developmental study. Collected anthers were air-dried at room temperature for 24 h, then wrapped in absorbent filter paper, sealed in a clear Ziploc bag, and stored at –4 °C. Although the flowers had not opened, they

contained both dehisced and undehisced anthers. A voucher specimen (KuangYan-Feng 1002) is preserved at South China Botanical Garden Herbarium IBSC.

To prepare unacetolysed samples, dehisced and undehisced anthers were dissected out of unopened flowers, separated and put into separate plastic vials in 70% ethanol. The anthers were then squeezed with forceps to release the pollen. The separation of the pollen grains from the remaining anther material was accomplished with a steel sieve (mesh diameter 100 μm). The separated pollen was rinsed in 70% ethanol with ultrasonic vibration for 30 min and centrifuged (4000–5000 RPM, 5 min) to create a pellet. The ethanol was decanted and fresh 70% ethanol was added. The samples were stored in 70% ethanol prior to decanting onto stubs, air drying and observation with a scanning electron microscope.

For acetolysis, undehisced anthers were placed in plastic vials, covered with glacial acetic acid, squeezed to release the pollen and filtered as described earlier. The pollen was subdivided into four subsamples, all of which were centrifuged separately to create pellets. The glacial acetic acid was decanted and a mixture (9:1) of acetic anhydride and concentrated sulphuric acid was added to each subsample. The four subsamples were acetolysed in a hot water bath at c. 90 °C for 1.5 min, 3 min, 5 min and 10 min, respectively. After acetolysis, the samples were cooled at room temperature, and centrifuged again at 4000–5000 RPM for 5 min. The residue of the chemical mixture was decanted and the pollen samples were rinsed in an ultrasonic bath with two changes of 70% ethanol, for 30 min each. The treated samples were stored in 70% ethanol prior to decanting onto stubs, air drying, and observation.

For scanning electron microscopy (SEM), the acetolysed and unacetolysed grains were mounted on small pieces of cover slip that were attached to SEM stubs with a strip of double-stick conductive tape. The pollen suspension was removed from the bottom of the plastic vials with a Pasteur pipette, placed on the cover glass and air-dried. Colloidal silver paint (#12630, Electron Microscopy Sciences, Fort Washington, PA, USA) was used to paint strips from the edges of the cover slip to the side of the stub. These strips conduct electrons from the top of the cover slip to the stub. Each sample was then sputter-coated with gold for 4 min (two coatings of 2 min each) in a PELCO Model 3 Sputter Coater 9100. A tilted stub holder was used for the second coating. Digital images were captured with a Hitachi S-4800 scanning electron microscope.

For light microscopy (LM) and transmission electron microscopy (TEM), fresh flowers of Uncaria hirsuta at different stages were collected from living plants growing in the South China Botanical Garden and the Dinghushan National Natural Reserve (Zhaoqing, Guangdong Province, China). Plants at both sites are cultivated. Voucher specimens (Kuang Yan-Feng 1002 from South China Botanical Garden, Kuang Yan-Feng 002 from Dinghushan) are deposited at IBSC. The fresh flowers were immediately fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.2, in the field. When back in the laboratory, the materials were placed under vacuum for 2 h and stored at 4 °C for several days. After removal from storage, the anthers were extracted, placed in the same fixation fluid under vacuum for 2 h, rinsed in 0.1 mol/l phosphate

buffer for 2 h and postfixed in 1% osmium tetroxide overnight. Following postfixation, the anthers were washed in phosphate buffer, dehydrated in an acetone series, embedded in Spurr's resin and cured at 70 °C. Semi-thin sections (1–2 µm) were cut with glass knives on a LKB-11800 microtome, stained with 0.1% toluidine blue and observed and photographed with an Olympus-AX70 light microscope equipped with an Olympus-DP50 digital camera. Ultrathin sections (80 nm) were cut using a Leica-Ultracut S ultramicrotome with a diamond knife and stained with uranyl acetate and lead citrate. Transmission electron micrographs were taken with a JEM-1010 transmission electron microscope at 100 KV.

Results

Morphology of pollen and protruding onci at different developmental stages

Pollen grains of Uncaria hirsuta are oblate spheroidal in equatorial view, semi-circular in polar view, tricolporate with of long ectocolpi and lolongate to (sub)circular mesopori. The exine ornamentation might either be described as striate-reticulate with interwoven muri or as rugulate with slender, long striae (Figures 1A, B, 2B, F).
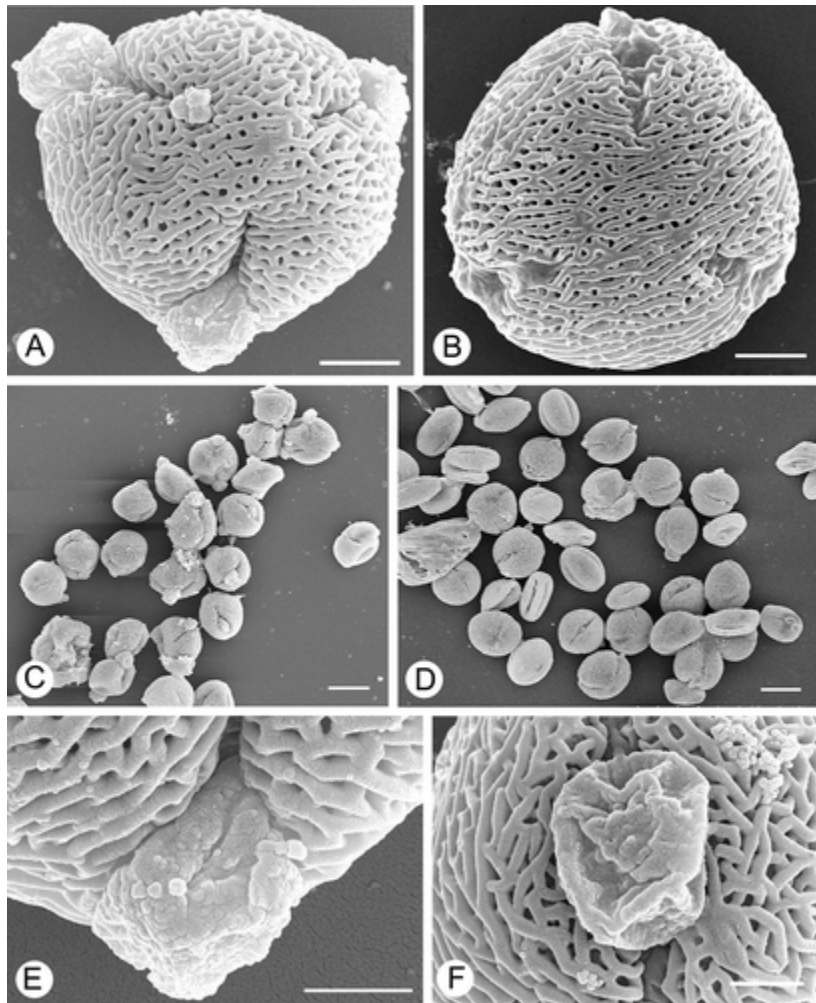
Figure 1. SEM of unacetolysed pollen grains of Uncaria hirsuta. A. Pollen grain from an undehisced anther, showing relatively rugulate muri, relatively narrow colpi and large protruding onci. B. Pollen grain from a dehisced anther, showing relatively indistinct protruding onci. C. Pollen grains from an undehisced anther. Protruding onci occur in most pollen grains. D. Pollen grains from a dehisced anther. Protruding onci occur only in hydrated pollen grains. E. Close-up of a protruding oncus; pollen from an undehisced anther. F. Close-up of a shrunken protruding oncus; pollen collected from an undehisced anther. Scale bars –10 μm (C, D), 2.5 μm (A, B), 1.5 μm (E, F).

Protruding onci are almost universally present in pollen grains collected from undehisced anthers (Figures 1C, 3A, B). Most are large and easily discernible under SEM and LM, even at low magnifications (Figures 1A, C, 3A). A few grains have small protruding onci and very few lack these structures (Figures 1C, 3A). When present, protruding onci usually emerge from the middle of the colpi.

The number of grains with protruding onci is much lower in dehisced (Figure 3C) than undehisced anthers (Figure 3A, B). When present in dehisced anthers, the protruding onci are smaller and less easily distinguished (Figures 1B, D, 3C). Large protruding onci occur in very few grains from dehisced anthers.

In undehisced anthers, the protruding onci are usually irregularly hemispherical, with a varying number of contortions on their surface (Figure 1A, E, F). In dehisced anthers, the protruding onci are smaller and more irregular and can easily be overlooked (Figures 1B, 2B, 3C).They occur either as a small protrusions extending above the aperture (Figures 2A, 3C) or as very small projections that extend only slightly, if at all, beyond the aperture (Figures 1B, 2B).
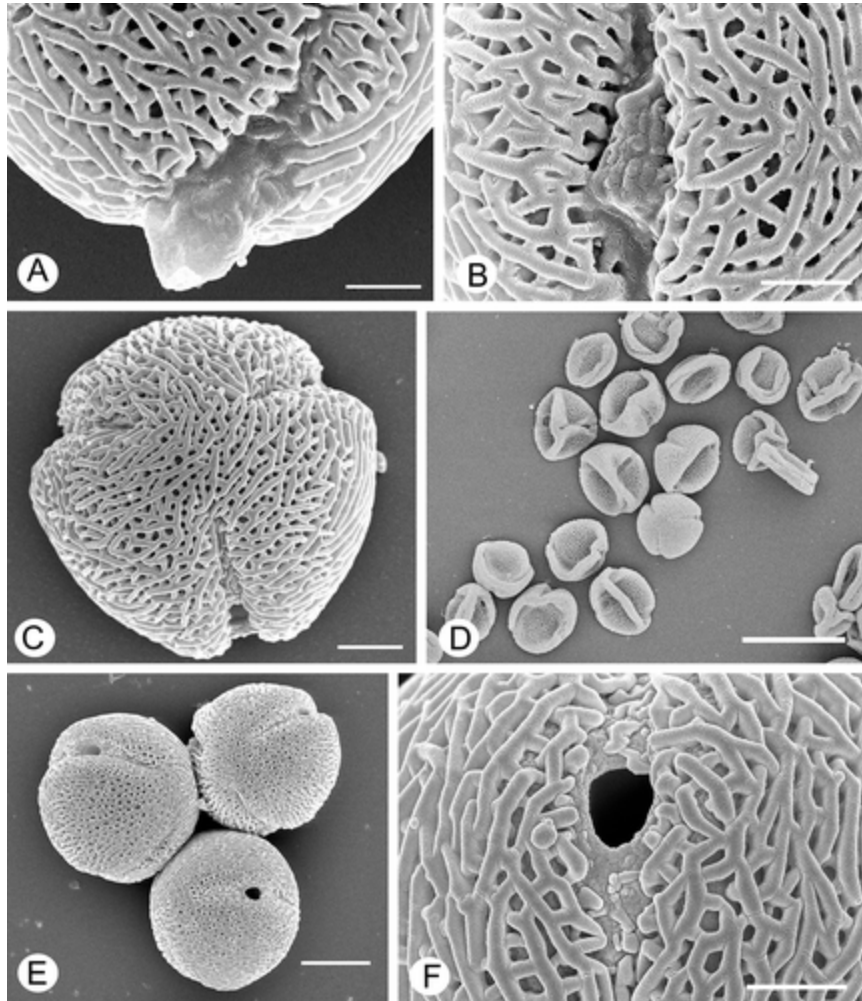
Figure 2. SEM of unacetolysed and acetolysed pollen grains of Uncaria hirsuta. A. Close-up of a relatively small protruding oncus from an unacetolysed grain; pollen from a dehisced anther. B. Close-up of a very small protruding oncus from an unacetolysed grain; pollen from a dehisced anther. C. Pollen from undehisced anther, acetolysed for 90 s; note the lack of protruding onci and the clear visibility of the mesopori. D. Pollen from an undehisced anther, acetolysed for 3 min; many pollen grains have collapsed. E. Three pollen grains from an undehisced anther, acetolysed for 10 min; note lack of protruding onci and the visibility of mesopori. F. Close-up of mesopori, pollen from undehisced anther, acetolysed for 10 min. Scale bars – 20 μm (D), 5 μm (E), 2.5 μm (C), 1.5 μm (A, B, F).
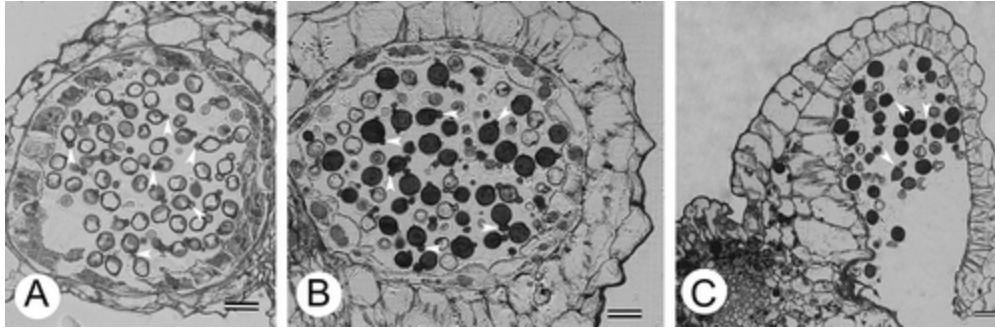
Figure 3. Light micrographs of semi-thin sections. A. Cross-section of an undehisced anther at the vacuolated microspore stage; large protruding onci (white arrowheads) occur as part of most microspores. B. Cross-section of an undehisced anther at the binucleate pollen stage; large protruding onci (white arrowheads) are still present in most pollen grains. C. Cross-section of a dehisced anther; small protruding onci (white arrowheads) are present in few pollen grains. Scale bars – 20 μm.

Acetolysis effects on protruding onci

Protruding onci are destroyed and the mesopori become conspicuous, following acetolysis treatments of 1.5 min or more (Figure 2C, E, F). After three or more minutes of acetolysis, some grains collapse (Figure 2D).

Microspore and protruding oncus development

At the tetrad stage, the microspores are enclosed by callose walls (Figure 4A). In the future aperture region, pro-endexine forms and becomes lamellated; large, lightly stained, electron-lucent areas form; and abundant vesicles accumulate at the junction of the cytoplasm and the electron-lucent areas (Figure 4A).
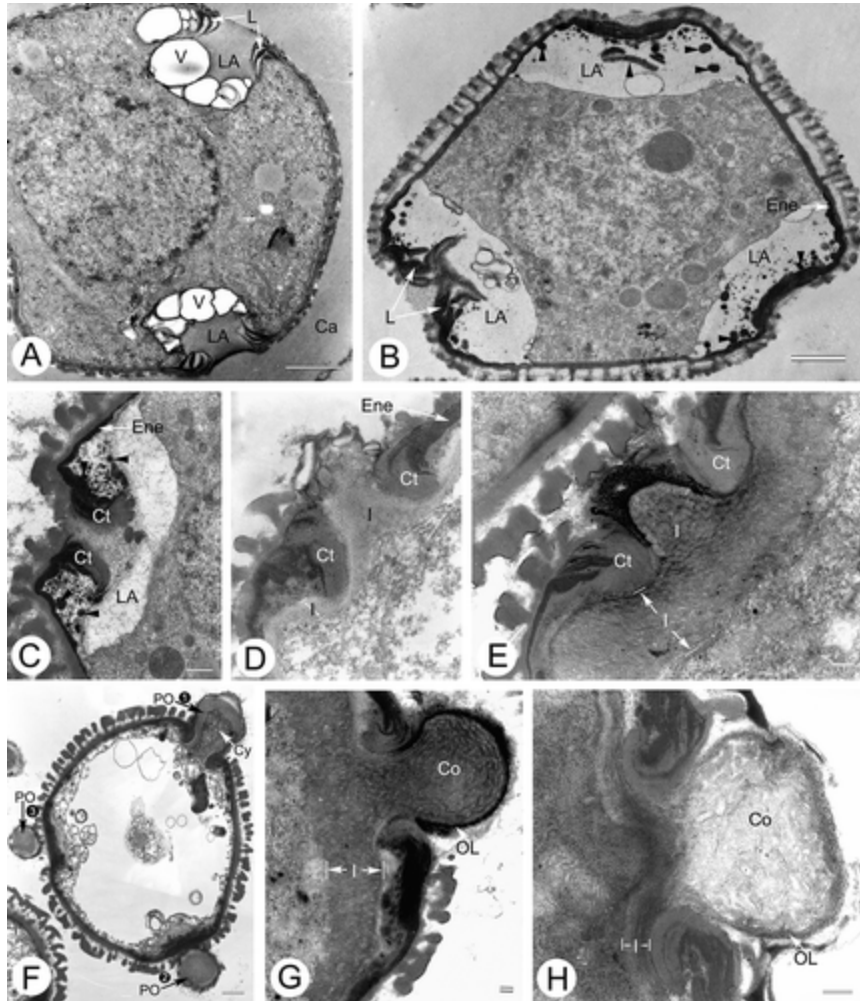
Figure 4. TEM of ultra-thin sections. A. Microspore at the tetrad stage enclosed by a callose wall (Ca); pro-endexine lamellae (L) and lightly stained and electron-lucent areas (LA) are visible below the proto-apertures; abundant vesicles (V) accumulate at the junction of the lightly stained areas and the cytoplasm. B. Early free microspore stage showing apertural lamellae (L) and electron-lucent lens-shaped areas (LA); abundant sporopollenin granules (black arrowheads) are distributed within the lens-shaped areas and attached to the surface of the lamellae; Ene – endexine. C. Free microspore stage showing flattened lens-shaped area (LA); endexine (Ene) around the aperture has thickened to form costae (Ct); sporopollenin granules (black arrowheads) are distributed around the costae (Ct). D. Vacuolated microspore stage; lens-shaped areas have disappeared and intine (I) deposition has begun; Ct – costae, Ene – endexine. E. Vacuolated microspore stage; intine (I) has thickened beneath the aperture and begun to extend through the colporus; Ct – costae. F. A vacuolated microspore showing three protruding onci (PO); the protruding oncus (1) is sectioned through the mesoporus; cytoplasm (Cy) is visible in its core; the cytoplasm in the protruding oncus is continuous with the cytoplasm in the body of the grain. G. Part of a binucleate pollen grain from an undehisced locule; protruding oncus is composed of an outer thin, electron-dense layer (OL) and an inner extremely thick core (Co); intine (I)

beneath the aperture is extremely thick. H. Part of a binucleate pollen grain from a dehisced locule; intine (I) below the aperture is thinner than in pollen from undehisced locules and the core of the protruding oncus is more electron-lucent. Scale bars – 1 μm (A, B, F), 500 nm (C); 200 nm (D, E, G, H).

At the early microspore stage, the callose wall dissolves and the free microspores are released into the anther locule. As the pollen grain increases in size, the electron-lucent areas broaden and become lens-shaped (Figure 4B). At this stage, electron-dense sporopollenin granules occur inside the electron-lucent areas. Some of these granules are attached to the surface of the inner lamellae, some are free in the electron-lucent areas and some are fused to the endexine (Figure 4B).

At the middle microspore stage, the electron-lucent areas flatten even more and the endexine thickens into costae around the aperture region (Figure 4C).

During the vacuolated microspore stage, the electron-lucent area disappears and intine deposition starts (Figure 4D). Intine formed in this region becomes extremely thick and protrudes through the aperture, thus forming protruding onci (Figure 4E, F). Protruding onci occur through all three apertures of a pollen grain at the same time and have similar sizes and morphologies (Figures 3A, B, 4F). Cytoplasmic contents sometimes extend into the protruding onci, perhaps due to the increase in size of the central vacuole of the microspore (Figure 4F). The final protruding onci consist of a thin, outer electron-dense layer and an extremely thick, electron-lucent core (Figure 4G).

In pollen grains from dehisced anthers, the intine beneath the aperture is thinner and the core of the protruding oncus is more electron-lucent (Figure 4H).

Discussion

Development of the protruding oncus

In this paper, we have elucidated the main stages of the formation of protruding onci in Uncaria hirsuta. At the tetrad stage, a lens-shaped area forms beneath the future aperture and determines the site of the protruding oncus. During the vacuolated microspore stage, the intine forms, the lens-shaped area flattens and the initine thickens beneath the aperture and protrudes outwards, forming the protruding oncus. At anther anthesis, the aperture intine is much thinner than at earlier stages and the protruding onci may hardly be visible. These changes are likely due to the harmomegathic effect, which occurs after anther anthesis.

The time of the onci formation is dependent on the taxon. The onci of Mitriostigma axillare Hochst. form at the tetrad stage, protrude through the aperture when the grains separate, contain only minimal cytoplasmic contents and are not shed from the grain (Hansson & El-Ghazaly,

2000). In contrast, the thick onci of Tarenna gracilipes (Hayata) Ohwi form in mature pollen grains just prior to dehiscence (Vinckier & Smets, 2005).

The effects of acetolysis and anther dehiscence on the protruding oncus

The acetolysis method introduced by Erdtman (1943) is a widely employed, successful technique in palynology. However, acetolysis destroys all pollen contents with the exception of the sporopollenin (Hesse & Waha, 1989). As a result, intinous structures like protruding onci are destroyed. Even 1.5 min of acetolysis treatment was enough to destroy the protruding onci in Uncaria hirsuta. As Hesse and Waha (1989) pointed out, the use of acetolysis may lead to the failure to report intinous structures and may even distort some aspects of pollen structure. For instance, breakdown of the intine, which supports the thin exine of Strelitzia reginae Banks, may result in faulty reports of pollen diameter, sculpturing and shape (Hesse & Waha, 1983, 1989).

The presence of protruding onci is also affected by the stage of anther dehiscence. Before anther dehiscence, protruding onci are almost universally present and conspicuously visible in the grains. At anther dehiscence, protruding onci are usually absent, and when present, are small and undistinguished. Hence, it is important to identify the developmental status of anthers (whether dehisced or undehisced) from which pollen grains are collected in order to verify the presence or absence of protruding onci.

Pollen buds versus protruding onci

The term 'pollen buds' was introduced to describe the spherical buds occurring outside of the germ pores of developing pollen grains in Ophiorrhiza mungos L. (Philip & Mathew, 1975). Since that time, pollen buds have been reported in Mussaenda (Priyadarshan & Ramachandran, 1984), Pseudomussaenda and Schizomussaenda (Puff et al., 1993) and in another ten species of Ophiorrhiza (Chennaveeraiah & Shivakumar, 1983; Mathew & Philip, 1987), which are all members of the Rubiaceae.

Weber and Igersheim (1994) suggested (in a footnote) that the term 'pollen buds' is inappropriate, but did not suggest an alternative. In Weber's (1996) following paper, she referred to the apertural intine protrusions of Geranium (Geraniaceae) as 'apertural chambers', but this term has not been used extensively in the later literature.

The term 'protruding oncus' was initially proposed to replace the term 'pollen bud' (Tilney & Van Wyk, 1997). However, since that time, several authors have suggested that protruding onci and pollen buds are distinct structures based on three criteria: pollen buds are usually larger than protruding onci, they usually possess a vacuole (Igersheim & Weber, 1993; Weber & Igersheim, 1994; Hansson & El-Ghazaly, 2000) and they are severed from the grains before shedding (Philip & Mathew, 1975; Chennaveeraiah & Shivakumar, 1983; Priyadarshan & Ramachandran, 1984; Weber & Igersheim, 1994; Dessein et al., 2005). In general, large, conspicuous vacuoles are not found in protruding onci, while cytoplasmic inclusions are occasionally present. For

example, Tilney and Van Wyk (1997) described cytoplasmic inclusions in the protruding onci of Canthium, Keetia and Psydrax (Rubiaceae), while Kuang et al. (2008) reported cytoplasmic contents in the protruding onci of Uncaria hirsuta.

In the Rubiaceae, Ramam (1954) and Farooq and Inamuddin (1969) were the first to report intines protruding from the apertures. Ramam (1954) reproduced the protrusions only in drawings based on LM and did not describe their internal structure or fate. Farooq and Inamuddin (1969) reported that the intine protrudes through the aperture in Oldenlandia nudicaulis Roth., but did not illustrate this feature.

The most comprehensive investigation of protruding intinous structures in the Rubiaceae is in the Naucleeae (Kuang et al., 2008). Kuang et al. (2008) found that of the 12 investigated genera, only Mitragyna lacks these projections. Rubiaceae species with similar protrusions were also sometimes figured in publications, but were not formally described by the authors (e.g. Robbrecht, 1988, p. 116, figure 45F; Maheswari Devi & Krishnam Raju, 1990, p. 53, figures 24–26; Igersheim, 1991, p. 143, figure 4K; Igersheim, 1993, p. 557 figure 11A; Puff & Rohrhofer, 1993, p. 163, figure 11A, B, D; Huysmans, 1998, p. 123 figure, 3, p. 127 figure 11, p. 129, figures 22, 24; Cai et al., 2008, p. 66, figure 16). In some cases, these protrusions were designated by different names, viz. 'protruding intine' (Johansson, 1992, p. 47, figure 11E, F), or 'papillae' (Hansson & El-Ghazaly, 2000, p. 78, figure 41, p. 80, figure 48; Vinckier & Smets, 2005, p. 40, figure 8F, G).

In order to further investigate the extent of occurrence of apertural protrusions in the family Rubiaceae, the first author used SEM observations, preceded by fixation in glacial acetic acid, washing in 70% ethanol and air drying, to scan pollen samples of 229 species belonging to 67 genera of Chinese Rubiaceae. Fifty species belonging to 28 genera were found to carry this palynological feature. Twenty-one of these genera were found to have this feature for the first time, viz. Aidia, Alleizattella, Diplospora, Dunnia, Duperrea, Fagerlindia, Hemalia, Himalrandia, Houstonia, Hyptianthera, Ixora, Leptomischus, Litosanthes, Morinda, Mycetia, Myrioneuron, Neanotis, Neohymenopogon, Oxyceros, Porterandia, Tarenna (Kuang, unpublished data, 2011). According to these preliminary observations, supplemented with a literature review, the occurrence of apertural protrusions in the Rubiaceae was found to have a tentative correlation with the number of apertures. In most cases, the protrusions appear in three or four aperturate pollen grains. Grains with fewer, or more, apertures lack them. An exception appears to be Mussaenda glabrata Hutch., where Priyadarshan and Ramachandran (1984) reported that pollen grains with four, five and even six protruding onci were found, a finding that also suggests that there are a variable number of apertures in this species. Though we cannot be certain that these protrusions are all derived from the intine, it seem likely that they are based on Kuang et al.'s (2008) finding that intine protrusions are relatively common in the Rubiaceae.

Outside the Rubiaceae, apertural protrusions have been reported in Forsythia suspensa Vahl (Oleaceae: Cao & Wang, 1992), Geranium (Geraniaceae: Weber, 1996), Pelargonium

(Geraniaceae: Hu & Zhu, 2000; Hu, 2005) and Oenothera (Onagraceae: Noher de Halac et al., 1990; Takahashi & Skvarla, 1990; Noher de Halac et al., 1992). They were referred to by different names in these studies, but appear to have similar structures. Cao and Wang (1992) referred to the structure in Forsythia suspensa as an 'intinous oncus.' Noher de Halac et al. (1990) referred to the corresponding structure in Oenothera as an 'apertural chamber,' the same name as used by Weber (1996) in Geranium. Hu and Zhu (2000) and Hu (2005) referred to it as a 'cytoplasmic bulge' in Pelargonium.

Without the information available from TEM studies, it is difficult to clearly determine if an apertural protrusion is a pollen bud, a protruding oncus or some other type of protrusion that does not involve the intine. Although, protruding intines may occur frequently, they may not be reported in the literature (see Dessein et al., 2005; Kuang et al., 2008). Knowing the full extent of their occurrence may help clarify the difference, if any, between pollen buds and protruding onci. It may be that the difference is merely a matter of degree, with pollen buds containing more extensive vacuolar and occasionally cytoplasmic contents than the protruding onci and thus having a larger size (Hansson & El-Ghazaly, 2000; Dessein et al., 2005). The suggestion that pollen buds are shed while protruding onci are not, is dealt with below.

Weber (1996) noted that some apertural intine protrusions are separated from the main cytoplasmic contents of the pollen gain by a layer of intine and are shed from the grain, while others are not separated by a layer of intine and are retained on the grain. This suggests that a difference among protrusions may be the presence of this layer and the fact that some protrusions are shed. However, the protruding onci of Adina pilulifera Franch. ex Drake, Metadina trichotoma Bakh. f., Neolamarckia cadamba Bosser and Pertusadina hainanensis Ridsd. (all Rubiaceae) lack cytoplasmic contents and are all shed (Kuang et al., 2008). In these grains, the protruding onci consist only of intine and there is, therefore, an intinous layer separating the bulk of the protruding oncus from the main body of the pollen grain. This suggests that Weber's (1996) distinction may not hold up when the structure of intinous protrusions is known in a wider range of taxa.

Hansson and El-Ghazaly (2000) have a different interpretation of the feature that is associated with protrusions that are shed. According to these authors, those protrusions with cytoplasmic contents are shed from the pollen grain. However, Kuang et al. (2008) demonstrated that shedding of the protrusions is independent of the presence of vacuolar and/or cytoplasmic contents. They found protruding onci that lacked vacuolar and/or cytoplasmic contents and were either shed or remained attached to the pollen grain, depending on the taxon. The situation in Uncaria hirsuta illustrates the weakness of Hansson and El-Ghazaly's (2000) suggestion. The protruding onci of U. hirsuta possess cytoplasmic contents, but are retained on pollen grains.

Additional study on the occurrence, ultrastructure and development of protruding intinous structures of a wider range of taxa should help clarifying this issue and elucidating the difference, if any, between pollen buds and protruding onci.

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