Vesicular cells of the lodgepole pine dwarf mistletoe (Arceuthobium americanum) fruit: development, cytochemistry, and lipid analysis

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Abstract: The lodgepole pine dwarf mistletoe, Arceuthobium americanum Nutt. ex Engelm., is a parasitic angiosperm that infects conifers in western Canadian forests. A striking feature of the Arceuthobium life cycle is explosive seed dispersal, triggered by hydrostatic build-up within the fruit, localized in a tissue called “viscin.” Viscin is composed of two cell types: “viscin cells” that accrue pressure in their hydrated mucilage, and “vesicular cells” with a previously undetermined function. The objective of this work was to investigate the development and composition of vesicular cells using microscopy, cytochemistry, and gas chromatography – mass spectroscopy. We found that vesicular cells are initiated from mesocarp cells lining the viscin cells. As development of the seed progresses, vesicular cells form a single layer, enlarge, and become isodiametric. Later, the cells make up all of the remaining 3–4 outer mesocarp layers. Concomitant with enlargement, intracellular triacylglycerides (TAGs) largely composed of 16-carbon and 18-carbon fatty acids, accumulate. Immediately prior to discharge, vesicular cell boundaries become indistinct, and the cell contents become confluent, creating a lipid mass between the viscin cells and the exocarp. Vesicular cells and their resulting lipid mass likely function in discharge by acting as a hydrophobic barrier to water loss and (or) as a repellant, resistant layer.

Key words: cytochemistry, dwarf mistletoe, gas chromatography – mass spectroscopy (GC-MS), triacylglycerides (TAGs), viscin tissue.

Résumé : Le gui nain du pin lodgepole, Arceuthobium americanum, est une angiosperme parasite infectant les forêts de l’Ouest canadien. On y observe une caractéristique bien particulière, soit la dispersion explosive des graines, déclenchée par la formation d’une pression hydrostatique à l’intérieur du fruit, localisée dans un tissu “vicinal.” Ce tissu comporte deux types de cellules: les “cellules vicinales” qui augmentent la pression dans leur mucilage hydraté et les “cellules vésiculaires”, dont la fonction n’a encore jamais été définie. Les auteurs ont étudié le développement et la composition des cellules vésiculaires à l’aide de la microscopie, de la cytochimie et de la chromatographie en phase gazeuse – spectroscopie de masse. Ils ont constaté que les cellules vésiculaires prennent naissance dans les cellules du mésocarpe bordant les cellules vicinales. Au cours du développement de la graine, les cellules vésiculaires forment une seule couche, grossissent et deviennent isodiamétriques. Plus tard, les cellules transforment toutes les cellules résiduelles des 3–4 couches du mésocarpe externe. De façon concomitante avec l’agrandissement, elles accumulent des triacylglycérides (TAGs) constitués surtout d’acides gras à 16 et 18 carbones. Immédiatement avant la décharge, le pourtour des cellules vésiculaires devient indistinct, et les contenus des cellules deviennent confluentes, créant une masse lipidique entre les cellules vicinales et l’exocarpe. Les cellules vésiculaires et la masse lipidique qui en résulte interviennent vraisemblablement dans la décharge, en agissant comme couche de résistance sous forme d’une barrière hydrophobe à la perte de l’eau ou comme répulsif.

Mots-clés : cytochimie, gui nain, chromatographie en phase gazeuse – spectrographie de masse (GC-MS), triacylglycérides (TAGs), tissu vicinal.

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Introduction

Mistletoes are flowering plants and aerial parasites belonging to the order Satantales (Kuijt 1968; Cronquist 1981; Mathiasen et al. 2008). The mistletoe habit is believed to have evolved five times independently in the Satantales (Der and Nickrent 2008; Mathiasen et al. 2008); while the order is in a state of taxonomic revision, the classic mistle-
two growing seasons to fully develop (Hawksworth and Wiens 1996). The *Arceuthobium* fruit, with its single, integumentless seed, has several intriguing characteristics. Arguably though, the most noteworthy of these features is its mode of seed dispersal: the fruit’s atemic seed is explosively discharged from the mature fruit of essentially all members of the genus aside from *A. verticilliflorum* Engelmann. Ballistic discharge is a hallmark of this genus; birds typically disperse the seeds of other mistletoes.

Forcible seed discharge in *Arceuthobium* is thought to be accomplished by way of a hydrostatic pressure that builds up in a mucilaginous tissue called “viscin” (Hawksworth and Wiens 1996). Ross Friedman and Sumner (2009) confirmed that the viscin tissue of *A. americanum* represents the mesocarp, and is a complex tissue consisting of two cell types: viscin cells and vesicular cells. Viscin cells, which are elongated and secrete a hydrophilic mucilage, comprise the bulk of this tissue in *A. americanum*. The pressure required for discharge accumulates within the extracellular mucilage of the viscin cells, which are found attached to the modified endocarp that envelopes the atemic seed following discharge. Viscin tissue, including both viscin cells and vesicular cells, are found in other mistletoes (see Sallé 1983; Gedalovich and Kuijt 1987).

In *A. americanum*, mature vesicular cells are isodiametric, containing a large hydrophobic component, and are found external and adjacent to the viscin cells (Ross Friedman and Sumner 2009). Relative to the cells of the exocarp, vesicular cells seem enlarged and swollen. Vesicular cells remain with the fruit exocarp following discharge, and their function is obscure. Sallé (1983) described “vacuolated” cells in *Viscum album* L. (Viscaceae), which are found at the outer periphery of the viscin cell layer, and have the same general appearance as the vesicular cells in *A. americanum*. Ross Friedman and Sumner (2009) suggested that these vacuolated cells are synonymous with the vesicular cells described for *A. americanum*. Gedalovich and Kuijt (1987), who coined the term “vesicular cells”, observed them in the Loranthaceous mistletoe *Phthirusa pyrifolia* (H.B.K.) Eichler. However, vesicular cells in this species are slightly more elongated than those in *A. americanum* and are interspersed with the viscin cells rather than being located on the periphery of the viscin cell layer. Studies on vesicular cells are limited. Ross Friedman and Sumner (2009) noted the point in development when the vesicular cells became more prominent, but there have been no published investigations into the origin and development of the vesicular cells in any mistletoe.

In addition, the vesicular cells in *Viscum album* L. (Sallé 1983) and *P. pyrifolia* (Gedalovich and Kuijt 1987) do not show evidence of being hydrophobic like those of *A. americanum* (Ross Friedman and Sumner 2009). As *Arceuthobium* species are dispersed explosively, perhaps the hydrophobic nature of the vesicular cells in the dwarf mistletoes when compared to the bird dispersed mistletoes is important. Furthermore, no previous study has determined the identity of the hydrophobic material.

The objectives of this work are (i) to carefully describe the origin and development of the vesicular cells of *A. americanum* using modern techniques of microscopy; and (ii) to employ cytochemical and biochemical techniques to elucidate the type(s) of hydrophobic material that is found in the vesicular cells. In turn, this information may further our knowledge of the life cycle of *A. americanum* and may offer an insight into the mechanism of seed dispersal in these important forest pathogens.

**Materials and methods**

**Site description, collection, and initial sample preparation**

The research site, dominated by lodgepole pine trees heavily infected with *A. americanum*, was located adjacent to Stake Lake (50°31′N, 120°28′W), 30 km south of Kamloops, British Columbia. Pistillate aerial shoots of *A. americanum* representing a range of developmental stages were photographed at the site with a Nikon E995 3.34 mp camera (Nikon, Tokyo, Japan) and collected twice weekly over the growing season (1 May 2008 through 30 August 2008). All of the samples had fruit that were in their second year of development. On average, about 2 shoots were collected each time, for a total of about 60 shoots obtained over the entire collection period; each shoot contained at least 5 fruits, and thus 300 fruits were obtained. All of the samples were taken from the host trees at about 1.5 m above the ground, as close to the trunk or branch as possible.

At the site, most shoots were placed in a fixative consisting of 2% (w/v) paraformaldehyde + 2% (w/v) glutaraldehyde in 0.1 mol/L phosphate buffer (pH 6.8). The material was kept at 4 °C overnight, then rinsed in the same buffer. For transmission electron microscopy (TEM), 1 of the 2 fixed shoots collected per sample date was postfixed with 2% (w/v) osmium tetroxide in the same buffer for 4 h. All fixed material was then dehydrated in an ethanol series and embedded in Spurr’s resin (Spurr 1969). Some of the samples taken from 16 June 2008 and 16 August 2008 were not fixed at the site but were instead stored at −80 °C.

**Staining of sectioned fruit for brightfield light microscopy**

About 30 sections from each non-osmicated fruit (1 median; 29 nearly median × 5 fruit for a total of 5 median and 145 nearly median sections per date) 2 μm thick were obtained with a Sorvall Porter-Blum JB-4 microtome (Sorvall, Newtown, Connecticut, USA) and affixed to gelatin-coated glass slides (Jensen 1962). The slides were subjected to the following brightfield light-level cytochemical staining procedures. Stained sections were viewed with a Nikon Eclipse E400 light microscope and photos taken using a Nikon E995 3.34 mp camera (Nikon, Tokyo, Japan).

**Toluidine Blue O**

For routine staining to highlight general morphology, as well as to localize phenolics and pectic acids, at least 5 sections per date (including median sections for determining dimension) were stained with 0.05% (w/v) Toluidine Blue O (C.I. 52040, Sigma) in a 0.05 mol/L benzoate buffer (pH 4.4). Under these acidic conditions, phenolics such as tannins stain blue-green, while pectins stain pink (Jensen 1962; O’Brien et al. 1964; Sumner 1988).
Periodic acid – Schiff’s treatment

A periodic acid – Schiff’s (PAS) treatment was used to localize insoluble carbohydrates, which stain pink under this regime (O’Brien and McCully 1981). Slides containing at least 5 sections per date were incubated in saturated 2,4-dinitrophenyl hydrazine in 15% (v/v) periodic acid for 30 min to block endogenous aldehyde, immersed in 1% (w/v) periodic acid for 30 min to oxidize vicinal 1,2 glycol groups to aldehydes, subjected to Schiff’s reagent for 45 min, and rinsed with distilled water (distilled water was substituted for periodic acid in the controls).

Aniline blue black stain

Aniline blue black (ABB) stain was used to localize protein following the methods outlined by O’Brien and McCully (1981). Slides containing at least 5 sections per date were placed into 1% (w/v) ABB (C.I. 20470, Polysciences, Warrington, Pennsylvania, USA) in 7% (v/v) acetic acid for 15 min at 65 °C. The slides with sections were rinsed in 7% (v/v) acetic acid and then mounted in glycerol containing 5% (v/v) acetic acid. A positive reaction results in a bright to dark blue stain, whereas a negative reaction results in no colour to a dull grey appearance. The ABB procedure was also used to counter stain PAS-treated sections (at least five per date).

Sudan Black B stain

Sudan Black B (SBB) stain was prepared using the method outlined in Ross (2002) as modified from Bronner (1975). The stain, 0.3% (w/v) of SBB (C.I. 26150, Sigma; sigmaaldrich.com) in 70% (v/v) ethanol, was heated to 37 °C for 14 h, and filtered twice. Prior to staining, slides were incubated in 70% (v/v) ethanol for 2 min, and then placed into stain at 55 °C for 1 h, rinsed in 70% (v/v) ethanol for 1 min followed by 15 min in 80% (v/v) ethanol at room temperature. When viewed, hydrophobic materials such as lipids stain black (Bronner 1975; Kiernan 2007).

Luxol Fast Blue stain

A solution of 0.1% (w/v) Luxol Fast Blue (LFB) was prepared using 95% (v/v) ethanol (Kiernan 2007). The slides were heated to 56 °C for 16 h, rinsed with 95% ethanol, and then distilled water. The slides were placed in a 0.05% (w/v) lithium carbonate solution for 30 s followed by 30 s in a 70% (v/v) ethanol solution, rinsed with distilled water, counter stained with a 0.1% (w/v) crystal violet solution, and rinsed with 95% ethanol. The LFB treatment stains phospholipids blue (Kiernan 2007).

Preparation of vesicular cell smears from unfixed samples and subsequent staining with Nile Red for fluorescence microscopy

Whole fruit stored at −80 °C (about five fruit per two sampling dates) were brought to room temperature, placed on gelatin-coated slides, and manipulated under a Kyowa Optical SDZ-AL dissecting microscope (Kyowa Optical, Tokyo, Japan). The seed and viscin cell coating was squeezed out and discarded. The remaining outer fruit shell was cut open to reveal the vesicular cells layers, which were removed with a scalpel and smeared onto the slides and left to air-dry.

A staining solution of Nile Red was made by adding 2–10 µL of a 500 µg/mL stock solution (oxidized from Nile Blue A, C.I. 51180, Sigma) to 1 mL of 75% (v/v) glycerol (Greenspan et al. 1985). A drop of the stain was placed on the slides, which were left to incubate for 5 min. Under 450–500 nm band excitation, neutral lipids, primarily triacylglycerides (TAGs), appear yellow-gold in color (528 nm band emission in acetone). Under 520–580 nm band excitation, phospholipids, amphipathic lipids, and strongly hydrophobic proteins appear red (570–620 nm emission). All samples were viewed with a Nikon Eclipse E400 light microscope fitted with a Nikon episcopic fluorescence attachment. Brightfield light microscopy was also used to ensure that the vesicular cells had been correctly identified.

Transmission electron microscopy

For TEM, about 5 ultrathin sections 50–90 nm in thickness (grey to gold interference colours) were obtained from each osmicated fruit (5 sections × 5 fruit per date) with a Reichert-Jung Ultracut ultramicrotome (Reichert-Jung, Buffalo, New York, USA) and adhered to the shiny side of uncoated copper hex grids (200 mesh). The sections were stained with saturated uranyl acetate in 50% methanol, and counterstained with 0.1% aqueous lead citrate. All sections were observed and photographed with a Hitachi Model H-7000 TEM (Hitachi, Tokyo, Japan) at an operating voltage of 75 kV.

Gas chromatograph-mass spectroscopy analysis of vesicular cell contents

The seeds and viscin cell coating of about 10 previously-frozen A. americum fruit collected 16 August 2008 were removed and discarded, and the remaining outer fruit shells, which contained the vesicular cell layers, were weighed and transferred to a microfuge tube containing 1 mL of hexanes. The fruit shells were macerated to separate the intracellular lipids from the membrane bound lipids, and centrifuged for 45 s at 5500 r/min (7500g). The supernatant, which contained the vesicular cell extract, was removed and transferred to a new microfuge tube and dried. The residual nonvesicular fruit tissue in the pellet was retained in the first tube.

To saponify and methylate the fatty acid (FA) acyl groups, 1 mL of Meth-Prep II® (Alltech, Deerfield, Illinois, USA) was added to both tubes. Then, 0.5 mL of hexanes was added to each tube, followed by centrifugation for 30 s. The hexane portion was removed, and the derivatized samples were analyzed using a Saturn 2200 GC-MS (Varian, Palo Alto, California, USA). The samples were injected (1 µL; split ratio 1/200) into a 30 m × 0.25 mm internal diameter (VF-5MS) column with a starting temperature of 150 °C, increasing at 5 degrees/min (helium carrier). Fragments with a m/z ratio of 50 to 500 were monitored. For comparison, standard fatty acid methyl esters (FAMEs), derived from FAs ranging from 14 to 18 carbons (GLC-80; Supelco, Bellefonte, PA, USA), were run, and the National Institute of Standards and Technology (NIST) mass spectral search program (version 1.7) was searched (NIST 1999). The GC-MS experiment was conducted in duplicate.
Results

Fruit development and overview of vesicular cells in the nearly-mature fruit

The fruit of *A. americanum* develops over the second season post-fertilization, and enlarges from May (Fig. 1) through June (Fig. 2) into July, becoming recurved as the end of the growing season approaches (Fig. 3). Mature vesicular cells examined about one month prior to explosive discharge (early August) are isodiametric, have a diameter of approximately 40 μm, and are large relative to the exocarp cells (Fig. 4). Vesicular cells are found in about 3–4 conspicuous cell layers in the upper three-quarters of the fruit, immediately outside of the viscin cells. Vesicular cell contents do not stain with Toluidine Blue O.

Origin, development, and cytochemistry of the vesicular cells

The mesocarp is about 4–5 cell layers thick at the beginning of the second growing season (early- to mid-May). At this time, the innermost uniseriate layer of mesocarp begins to differentiate into the viscin cells (Figs. 5 and 6). The outer 3–4 layers of mesocarp have prominent nuclei (Fig. 6), and are somewhat flattened radially; these are the “vesicular initials” — cells destined to develop into the vesicular cells. Aside from the nuclei, which react positively to the ABB stain for protein, the contents of vesicular initials do not show a positive staining reaction when subjected to a combined PAS (insoluble carbohydrate) – ABB (proteins) procedure (Fig. 6), nor do they stain positively for the identification of hydrophobic materials when treated with SBB (not shown).

Vesicular initials retain their appearance and staining reactivity until the end of May – early June: at this time, they still do not stain positively when treated with either PAS (Fig. 7) or ABB (Fig. 8), but the innermost layer of vesicular initials in the upper quarter of the fruit now reacted positively for hydrophobic materials when stained with SBB (Fig. 9), indicating that differentiation into the enlarged vesicular cells is imminent. Within the course of a few days, a wave of SBB-positive staining proceeds basipetally in the innermost layer of vesicular initials, signaling that this inner layer of vesicular cell initials in the upper three-quarters of the fruit is poised to initiate differentiation. All remaining outer mesocarp layers/vesicular initials rapidly become SBB positive as well.

By early- to mid-June, the inner layer of vesicular initials in the upper quarter of the fruit — the first that displayed a positive reaction to SBB — have become vesicular cells, as they have enlarged to an average diameter of about 30 μm (Fig. 10). The single layer of vesicular cells remained PAS negative, but strongly SBB positive (Fig. 11). Aside from their cellular boundaries, they were also negative for LFB, which suggests that their contents are not composed of phospholipids (Fig. 12). When treated with Nile Red and viewed with the excitation/emission filter pairing of 450–500/528 nm, the vesicular cells in whole mount smears fluoresced yellow-gold, which is a positive identification of TAGs (Fig. 13). Conversely, when treated with Nile Red and viewed with the excitation/emission pairing for phospholipids, amphipathic lipids, and strongly hydrophobic proteins, no fluorescence was evident (not shown). A few viscin cells and associated mucilage was evident when viewed under brightfield LM (Fig. 14), where it is clear that only the vesicular cells were fluorescing when stained with Nile Red and observed with the filter pairing for TAGs (compare Fig. 14 with Fig. 13). When viewed with TEM, vesicular cells show a large osmiophilic deposit, as well as several smaller peripheral osmiophilic bodies, and the cell wall is a typical primary wall (Fig. 15).
Throughout June and July, the vesicular cells in this single layer increased in size to their maximum diameter of about 40 μm, but the results for all cytochemical staining reactions remained unchanged. The cell walls did not appear to change in appearance or dye reactivity at either the light or electron level of microscopy, staining pink with Toluidine Blue O (not shown) and PAS (Fig. 10), blue with ABB (not shown), and not staining with either SBB (Fig. 11) or LFB (Fig. 12). In early August, the remaining outer 2–3 layers of mesocarp (the remaining vesicular initials) rapidly enlarged, following the same basipetal wave, so that all of the outer 3–4 cell layers of the mesocarp (the vesicular initials) in the
upper three-quarters of the fruit had differentiated into vesicular cells (Fig. 16). These cells were all filled with a highly hydrophobic component.

By mid- to late August, immediately prior to explosive discharge, an intriguing phenomenon occurred: the cell walls amongst adjacent vesicular cells apparently broke down or otherwise became indistinct, leaving a large SBB-positive deposit between the viscin cells and the cells of the exocarp (Fig. 17). Remnants of broken wall provide evidence for the breakdown.

**Gas chromatography-mass spectroscopy analysis of vesicular cell contents**

The residue from the vesicular cell hexanes extract had the consistency of an oily liquid. The GC-MS analysis of the derivatized vesicular residue yielded several peaks that had retention times within the range of the FAME standards. The largest peak eluted had a similar retention time and the same mass spectra profile as hexadecanoic acid methyl ester, derived from hexadecanoic acid (16:0; see Table 1). Other compounds present in the chromatogram had retention times and possessed mass spectra consistent with them being derived from octadecanoic acid (18:0), octadecenoic acid (18:1), and octadecadienoic acid (18:2) in the vesicular cell extract. The GC-MS analysis of the nonvesicular fruit cell extract, although we suspect that we have recognized many isomers, did not conclusively identify the FAs present in the vesicular cell development to the development in an amoeboid syncytial "lipid body," which is largely composed of TAGs (Shukla et al. 1998; Murphy and Vance 1999).

**Nature of the hydrophobic component of the vesicular cells**

Based on our results, and on the resemblance of vesicular cell development to the development in an amoeboid tapetum, we believe the hydrophobic component of the vesicular cells is largely composed of TAGs. Potential candidates for hydrophobic components of plant cells include TAGs, terpenes (volatile oils), phospholipids, and suberins, although typically only TAGs are found as large intracellular bodies (Murphy 1993; Fahn 2000; Zweytick et al. 2000). Our cytochemical evidence suggests that the SBB positive hydrophobic material within vesicular cells is largely composed of TAGs, as the Nile Red staining with the filter pairing for neutral lipid (essentially TAG) identification (Greenspan et al. 1985) showed a positive result, while the material did not stain with LFB, a phospholipid stain (Kiernan 2007).

The cytochemical results are supported by our GC-MS data, which indicate that FAs, making up the acyl groups of TAGs, are a key component of the vesicular cell extract. However, as isomers can exist for FAs, particularly regarding double-bond positioning in the unsaturated FAs, we cannot conclusively identify the FAs present in the vesicular cell extract, although we suspect that we have recognized likely candidates. The GC-MS results indicate that hexadecanoic acid (16:0) is apparently the predominant FA of the vesicular extract, with octadecanoic acid (18:0), octadecadienoic acid (18:2), and octadecenoic acid (18:1) also being present. These FAs are most likely incorporated into TAGs. This is not surprising, as FAs composing TAGs in plants and animals are most commonly 16, 18, and 20 carbons in length (Harwood 1997). Furthermore, hexadecanoic acid and octadecenoic acid are the most common FAs of plants:

**Discussion**

**Origin, development, and fate of the vesicular cells**

The vesicular cells of *A. americanum* begin developing about 2 weeks after the onset of viscin-cell development. Their development is characterized by cell enlargement, which is primarily achieved by the prolific accumulation of SBB positive, osmiophilic, intracellular material, as well as an increase in the number of cell layers from one to three or four. While many TEM sections were obtained, the large lipid component of the vesicular cells obscured the ultrastructural characteristics.

Immediately prior to explosive discharge, the vesicular cell contents become confluent, creating an SBB-positive deposit between the viscin cells and the cells of the exocarp. The fate of the vesicular cell walls, plasma membranes, and other cellular contents has not been determined; as we could not detect visible changes in the cell walls, the breakdown event must have been rapid. The formation of this hydrophobic SBB-positive mass is highly reminiscent of the late developmental stages of a typical amoeboid (syncytial) anther wall tapetum. Over the course of pollen development, the cells of the tapetum undergo apoptosis to form a large syncytial (or coenocytic) "lipid body," which is largely composed of TAGs (Shukla et al. 1998; Murphy and Vance 1999).
being the most common saturated unsaturated FAs, respectively (Cseke et al. 2006). It has also been reported that octadecadienoic acids are the most common polyunsaturated FA in plants, and octadecenoic acid is the FA with the highest molecular weight most common to plants (Harwood 1997).
Plant TAGs are almost invariably liquid at room temperature and thus considered “oils” (Lersten et al. 2006). The liquid consistency of the extruded vesicular cell extract coupled with the presence of the unsaturated FAs in the vesicular cell TAGs suggests that the vesicular TAGs are oils. The positive staining reaction of the vesicular cells with osmium tetroxide corroborates this suggestion, as osmium tetroxide reacts with unsaturated lipids (Lison 1960). We plan future work on the cellular origin of the lipids, as the TEM work here was not able to capture the dynamic aspects of lipid formation during vesicular cell development.

Relevance of vesicular cell structure and development: a discussion of function

In Arceuthobium, the seeds are explosively discharged, and there is no evidence of endozoochory (Hawksworth and Wiens 1996). Furthermore, while vesicular cells have been observed in the bird dispersed Viscum album (Sallé 1983) and Phthirusa pyrifolia (Gedalovich and Kuijt 1987), these vesicular cells do not have a hydrophobic component; in fact, Gedalovich and Kuijt (1987) stated that the vesicular cells may also serve as a specialized storage area for (water) soluble nutrients for the disseminator. Therefore, even if the vesicular cells of A. americanum shared an evolutionary history with those of other mistletoes, the function in bird dispersal has long been lost. While the formation of a contiguous vesicular cell lipid mass follows a similar pattern of development to that of an amoeboid tapetum, which serves to provide nutrition to developing pollen grains, such a deposit in the explosive fruit of A. americanum likely has function in roles other than as an energy source.

The vesicular cells, then, must have a key role in explosive discharge. As the hydrophobic vesicular cells essentially line the hydrophilic viscin cells, the developing vesicular cells could act as a seal to ensure that water accumulating in the viscin mucilage does not leak out, thus allowing for an increase in hydrostatic pressure. The increase in vesicular cell size and number of layers throughout development might be required to counter the force building in the region of the viscin cells. The final breakdown of the vesicular cell boundaries leading to the formation of a large TAG deposit, which occurs as discharge becomes imminent, may be needed to provide resistance to the impending explosion, and the contiguous TAG deposit, being oily, may contribute a hydraulic component to the building pressure. Furthermore, the degenerate vesicular cell layers and TAG deposit remains with the exocarp following discharge, which suggests that the degenerate layers and deposit might have functioned as an abscission layer or perhaps even as a hydrophobic repulsion with the hydrophilic viscin cell layer. Such repulsion would be based upon the polar energy of cohesion among water molecules (viscin cell mucilage) and hydrophobic attraction (TAG deposit) due to the hydrophobic effect (Van Oss 2006), and while not necessarily contributing to the explosive force within the fruit, would enable a particularly clean severance.

We plan to investigate the fruit and viscin tissue of two nonconformist mistletoes: Korthalsella sp. (Viscaceae), which is said to have mildly explosive fruit, and A. verticilliflorum, which is the only Arceuthobium species that does not explosively discharge its seeds (Hawksworth

<table>
<thead>
<tr>
<th>FAME</th>
<th>Hexadecanoic acid methyl ester</th>
<th>Octadecadienoic acid methyl ester</th>
<th>Octadecenoic acid methyl ester</th>
<th>Octadecanoic acid methyl ester</th>
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<td>Retention time (min)</td>
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<td>263, 150, 67</td>
<td>264, 222, 180, 55</td>
<td>227, 185, 171, 145, 74</td>
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<tr>
<td>Standard or NIST Library Sample</td>
<td>227</td>
<td>226</td>
<td>264</td>
<td>227</td>
</tr>
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</table>

Note: The vesicular cells were separated from the seeds, extracted in hexane, dried, methylated with Meth-Prep II (Alltech) and repartitioned in hexane; the identity of the FAMEs as analyzed by GC-MS was determined by comparing the retention times and mass spectra of the sample output with those of the standards and the NIST Library (NIST 1999).
and Wiens 1996). We expect to find vesicular cells in both mistletoes, but we expect those of Korthalsella to have features similar to those of A. americanum. Conversely, we suspect A. verticilliflorum vesicular cells may be divergent from those of A. americanum. Future work will also include further study of the viscin cells, the other component of the viscin tissue in all mistletoes. The uniqueness of the development, location, and oily content of the vesicular cells considered as a whole suggests that these cells are required for the process of explosive seed discharge in A. americanum: vesicular cells and their resulting lipid mass may act as a hydrophobic barrier to water loss and (or) as a repellent, resistant layer.

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