tion, when the radicle was 1 or 2 cm in length and metabolism was fully established. The seeds were then cut in half at the midline and processed as illustrated in Figure 1.

## Seeds used for fresh sections

Some of the seed halves were stored in water until sectioning on a cryostat. Others were stored in 70% alcohol, and others were fixed in FAA (10% formaldehyde/50% alcohol/5% acetic acid/35% water) for 72 h or in AGF (1.5% acrolein/3% glutaraldehyde/1.5% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) (Electron Microscopy Sciences, Hatfield, PA, USA) for 12 h. Then, the seed halves were washed with distilled water. Some halves were processed for paraffin embedding,[21] and others remained in water until sectioning.

## Histochemistry on fresh sections

The seeds that were stored in water, whether fixed or not, and the seeds that were stored in 70% alcohol were cut into 20-µm cross sections using a Leica CM1510S cryostat at between -14 and -18°C. Once on slides, the fresh sections were washed with distilled water three times for 5 min per wash to eliminate the Tissue Tech gel. The excess water near the section was absorbed with filter paper, and then the appropriate reagent was added. Kedde's reagent[8] (10% dinitrobenzoic acid and 2 N KOH in MeOH) is specific for ACGs, and a positive reaction yields a pink/magenta color. Lugol's iodine solution (0.5 g iodine and 2.0 g KI in 100 mL distilled water) yields a dark purple color when it reacts with starch. Sudan III (0.1% in EtOH)[18] yields a reddish orange color in the presence of fats. All analyses were performed at room temperature (approximately 23°C). After the reagents were applied, the samples were immediately observed through a microscope for ten to thirty minutes and photographed.

## Histochemistry on paraffin sections

The paraffin-embedded samples were cut into 8-µm sections using a Microm HM 340 E microtome (Thermo Fisher Scientific, Inc., Waltham, MA, USA), deparaffinised, rehydrated, stained for 10 min with Kedde's reagent to visualise the ACGs, and observed under a microscope. Other sections were rehydrated in 70% EtOH and stained with Naphthol Blue Black (1.0% in 50% EtOH) for protein analysis, and others were rehydrated in water, exposed to periodic acid (0.6%) for 1 min, and rinsed with distilled water. Then, Schiff reagent (Sigma-Aldrich 3952016; S. Louis, MO, USA) was added, incubated for 20 min and rinsed with distilled water. Then, the sample was dehydrated with xylene and mounted with resin. With this treatment, the polysaccharides stained a fuchsia color. Johansen's quadruple stain was added to sections that had been rehydrated in 70% EtOH. According to Johansen, although the mixtures are rather complicated, the procedure is simple; differentiation is automatic, and little is left to personal judgment.[21]

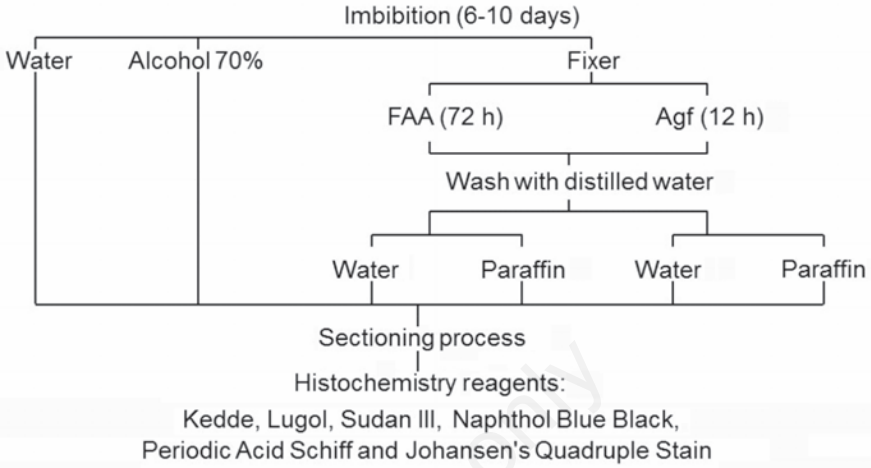


**Figure 1. Methods flowchart.**

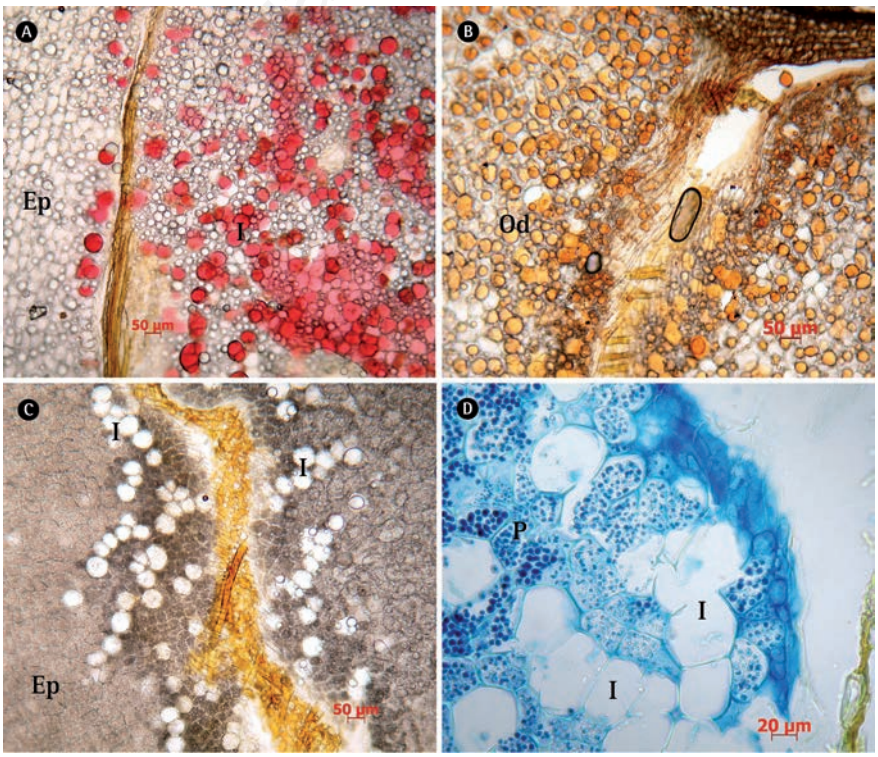


**Figure 2. A) Kedde's reagent positively reacts with the acetogenins in the idioblasts of fresh, unfixed sections, yielding a pink/magenta color. B) Sudan III positively reacts with oil drops to yield a reddish orange color in fresh, unfixed sections. C) Kedde's reagent does not react with fresh sections fixed in FAA; the idioblasts appear empty. D) Naphthol Blue Black reagent stains the cell walls and proteoplasts of paraffin sections blue, and the idioblasts appear empty. Ep, endosperm parenchyma; I, idioblasts; Od, oil drops; P, proteoplasts.**

### Microphotography

Photographs and idioblast measurements were taken with a Canon PowerShot A640 digital camera coupled to a Zeiss Axiostar Microscope with AxioVision software (Carl Zeiss Imaging Systems, Jena, Germany). Microscopic observations were made with A-Plan 10x/0.25 and A-Plan 40x/0.65 Ph2 objectives.

## Results and Discussion

Positive reactions with Kedde's reagent were only observed in fresh sections that were either unfixed or had been fixed in AGF and preserved in water, and staining was found only in the large cells (56×38 µm) at the periphery of the endosperm next to the tegmen; these cells are idioblasts (Figure 2A). The lipid nature of the ACGs was confirmed by their positive reaction with Sudan III in both fresh sections and sections that had been fixed in AGF (Figure 2B). Therefore, we propose that histochemical staining is appropriate for detecting ACGs and that the large cells are idioblasts that store ACGs.

Due to the use of solvents such as ethanol and xylene, none of the sections that had been fixed in FAA, stored in 70% alcohol, or embedded in paraffin showed staining in the idioblasts (Figure 2C). Naphthol Blue Black stained the cell walls blue, and the idioblast cytoplasm appeared secretory and lacked any special organelles; in the remaining endosperm cells, blue-stained proteoplasts were detected (Figure 2D) and might correspond to storage sites for albumin-type proteins, which have been reported to be stored in the endosperm of *A. muricata*[5] and *A. macroprophyllata*[22] seeds. Johansen's quadruple stain turned these organelles purple (Figure 3A), confirming the presence of protein reserves.

The presence of oils,[23] which act as reserve substances in the endosperm, was evident based on their reactivity with Sudan III (Figure 2B) and, indirectly, by the spaces observed in the cells in the paraffin-embedded material (Figure 3B). The material fixed in AGF, which had been preserved in water and sectioned on a cryostat, still contained ACGs in the idioblasts. The plastids were not stained by Schiff reagent (Figure 3C), demonstrating that they do not contain starch; their reaction with the crystal violet in Johansen's quadruple stain indirectly indicates that they contain proteins (Figure 2D).

Staining with Lugol's iodine (Figure 3D) did not indicate the presence of starch, but the reagent generated a golden brown color in the idioblasts in the same place as the reaction with Kedde's reagent, indicating the presence of dextrins.[5] However, the content appeared more like a drop-shaped liquid than the expected solid particles. This golden brown reaction product between the cytoplasmic content of the idioblasts and Lugol's iodine, which is similar in chemical composition to Wagner reagent,[24] may indicate the presence of alkaloids. Therefore, this reaction deserves further histochemical study; the biosynthesis of liriodenine, an aporphinic alkaloid, begins during the early stages of development of seedlings of *Annona macroprophyllata* Syn *A. diversifolia* Saff.[6]

ACGs are bioactive compounds with cytotoxic properties in multiple cell lines, and they have antitumour, antiparasitic, antimalarial, insecticidal, antimicrobial, antifungal and antibacterial effects.[15] In particular, the acetogenin laherradurin might act as an autoinducer or quorum-sensing signalling molecule that affects the expression of genes involved in biofilm formation in *Pseudomonas plecoglossicida* J26.[18] Since ACGs are located in idioblasts that are found only in restricted areas in the periphery of the endosperm, despite the large amount of protein and oil reserves contained in the endosperm of mature seeds,[25] ACGs may act as a barrier that protects seeds against insects and pathogens. In addition, the presence of alkaloids in the idioblasts might support this idea, because alkaloids are also strongly insect repellent and toxic to microorganisms.[17]

Villamil *et al.* reported amyloid granules in *A. muricata* endosperm.[5] However, in *A. macroprophyllata*, amyloids are not present because do not show blue or purple staining with Lugol's iodine in the endosperm cell walls, as reported by Kooiman[2] for 25 species of *Annonaceae*. Some endosperm cells in *A. macroprophyllata* stain brown, which may correspond to dextrins. As described by Kooiman,[2] the term *amyloid* refers to a group of polysaccharides with similar structures, particularly those reported by Schleiden in 1838 (in [2]) and by Vogel and Schleiden in 1839 (in [2]), who first used the term. Using Mitchell reagent, which has the same composition as Lugol's iodine, Kooiman detected cellulose and hemicellulose via blue staining in the presence of 75% sulphuric acid. Consistent with these ideas, we suggest that the term *amyloid* should no longer be used to refer to the group of compounds that can be detected with Schiff

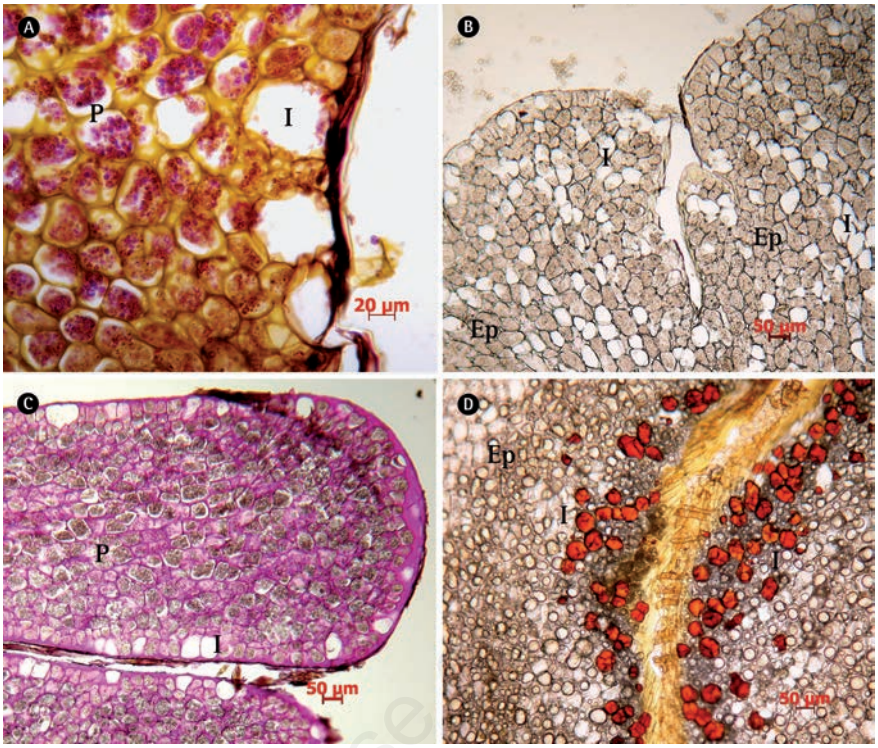


**Figure 3. A) Johansen's quadruple stain used on a paraffin section stains the proteoplasts purple. B) Kedde's reagent used on a paraffin section does not react, and the idioblasts appear empty. C) Schiff reagent used on a paraffin section stains the cell walls purple, but the proteoplasts and idioblasts do not stain. D) Lugol's iodine used on a fresh, unfixed section results in golden brown-colored drops in the idioblasts. Ep, endosperm parenchyma; I, idioblasts; P, proteoplasts.**

reagent,[21] as the term is now used to refer to a beta-fibrillar protein of animal origin, the amyloid beta-protein.[26]

This new histochemical method for detecting ACGs *in situ* in seed sections using Kedde's reagent can test for the presence of these substances in developing tissue or mature organs. It can also differentiate between various substances in the same structures, as it occurred using Warner reagent to detect alkaloids in idioblasts. This work histochemically demonstrates the compartmentalisation of ACGs in the endosperm, suggesting that its aforementioned characteristics are part of a protective strategy that allows selective defence for the sexual propagation of the species.

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