



Fast seed histology protocols: Benzene derivatives-free vs xylene-dependent¹

Protocolos de histología rápida de semillas: Libre de derivados de benceno vs dependiente del xilol

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Abstract

Introduction. Seeds are complex structures that allow the biological and crop propagation of plants. Seed histology can be used for teaching, researching, and for pathological diagnostic. Histology protocols are commonly divided into 5 different stages: fixation, processing, cutting, staining, and mounting. Xylene is a dangerous reagent used during the processing, staining, and mounting of histological specimens that can contaminate the environment and is toxic for users. **Objective.** To compare two new protocols for seed histology accelerated with microwave, tested on seeds of economic importance species. **Materials and methods.** The experiments were done between January and May of 2022 at the Centro de Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica. The compared protocols were: a benzene derivatives-free (BDF) and a xylene-dependent (XD). Seeds of *Carica papaya* L. (Caricaceae) var. Pococí, *Coffea arabica* L. (Rubiaceae) var. Obata, *Glycine max* L. (Fabaceae) var. CIGRAS-06, *Phaseolus vulgaris* L. (Fabaceae) var. Tayni, *Oryza sativa* L. (Poaceae) var. Lazarro FL, and *Zea mays* L. (Poaceae) var. EJN-2 were used. Three technical replicates of five seeds of each species were tested with the XD and BDF protocols, on different days each replicate. **Results.** The photomicrographs obtained with both protocols showed that the samples maintained the morphology integrity of embryo, endosperm or cotyledons, and other seed structures. BDF and XD protocols produced seed histology slides and microphotographs. PAS-Coomassie Blue staining made a good differentiation of carbohydrates and proteins. Fastness of both protocols is a benefit compared with other protocols for plant histology that can take several days or even weeks. **Conclusions.** The BDF and XD protocols were suitable for seed histology analysis of bean, coffee, maize, papaya, and soybean, slides were obtained in less than 5 hours. BDF protocol is the first for plant tissue processing that does not use benzene derivatives and that uses paraffin as embedding medium.

Keywords: periodic acid of Schiff, protein staining, microwave processing, Coomassie blue.



Resumen

Introducción. Las semillas son estructuras complejas que permiten la propagación biológica y agrícola de plantas. La histología de semillas puede ser utilizada para enseñanza, investigación y para diagnóstico patológico. Los protocolos de histología están divididos en cinco etapas: fijación, procesamiento, corte, tinción y montaje. El xilol es un reactivo peligroso que se usa durante el procesamiento, tinción y montaje de muestras histológicas que puede contaminar el ambiente y es tóxico para los usuarios. **Objetivo.** Comparar dos protocolos nuevos de histología de semillas acelerados con microondas, en semillas de especies de importancia económica. **Materiales y métodos.** Los experimentos fueron realizados entre enero y mayo del 2022 en el Centro para Investigaciones en Granos y Semillas de la Universidad de Costa Rica. Los protocolos comparados fueron: uno libre de derivados de benceno (BDF) y uno dependiente de xilol (XD). Se usaron semillas de *Carica papaya* L. (Caricaceae) var. Pococí, *Coffea arabica* L. (Rubiaceae) var. Obata, *Glycine max* L. (Fabaceae) var. CIGRAS-06, *Phaseolus vulgaris* L. (Fabaceae) var. Tayni, *Oryza sativa* L. (Poaceae) var. Lazarro FL, and *Zea mays* L. (Poaceae) var. EJM-2. Tres réplicas técnicas de cinco semillas de cada una de las especies se procesaron con los protocolos BDF y XD, cada réplica en días distintos. **Resultados.** Las fotomicrografías obtenidas con ambos protocolos mostraron que las muestras mantuvieron la integridad morfológica del embrión, del endospermo o de los cotiledones y de otras estructuras de la semilla. Los protocolos BDF y XD generaron láminas de histología y fotomicrografías. La tinción PAS- azul de Coomassie hizo una buena diferenciación de los carbohidratos y proteínas. La rapidez de ambos protocolos es un beneficio comparado con otros protocolos de histología vegetal que pueden durar varios días o incluso semanas. **Conclusiones.** Los protocolos BDF y XD fueron adecuados para el análisis histológico de semillas de frijol, café, maíz, papaya y soya en menos de 5 horas. El protocolo BDF es el primero de procesamiento de tejidos vegetales que no usa derivados de benceno e incorpora parafina como medio de inclusión.

Palabras clave: ácido peryódico de Schiff, tinción de proteínas, procesamiento con microondas, azul de Coomassie.

Introduction

Seeds are complex structures that contain an embryo, the energy reserve tissue, stored in the cotyledons, the perisperm or in the endosperm, that the embryo needs to germinate, and it is usually covered by a seed coat (Kaplan et al., 2009; Kelly et al., 1992; Rajjou et al., 2012; Sreenivasulu & Wobus, 2013; Waterworth et al., 2015). In nature, seeds allow plants to propagate, maintain its genetic heritage and keep the balance in the ecosystem (Rogers et al., 2021). In human society, seeds have a pivotal role in food safety because they are used to preserve and propagate crops species and varieties related to animal and human nutrition (Komarnytsky et al., 2022; Rajjou et al., 2012; Singh, 2016; Waterworth et al., 2015).

The histology and cytology of living organisms, including seeds, allow the understanding of basic microscopic anatomy and their role in physiological or pathological phenomena (Bracegirdle, 1977). In the case of seeds, histocytotechnological analysis is important for basic biology, teaching, research, and pathological diagnosis (Harris & Brolmann, 1966; Kaplan et al., 2009; Kshatriya et al., 2018; Mishra & Vijayakumar, 2014). To carry out these analyses samples must have the thickness of a cell diameter to be able to observe all their components on a microscope (McMillan & Harris, 2018). This implies that tissue samples need to be cut between 1 to 10 μm of thickness, depending on tissue type and on the histochemical technique to be applied. This is not possible with fresh tissue at room temperature because it is embedded in water that does not give enough stiffness to the tissue to be cut at a micrometric level (García, 1993; McMillan & Harris, 2018; Prophet et al., 1995).

Different types of resins are normally used to replace the water from the tissue and to increase its stiffness for a micrometric cut. There are polyester resins (Sidman et al., 2001), epoxy resins (Martin & Sofla, 2010) and paraffin (Alamri et al., 2017; Munganyinka et al., 2018), which is one of the most used resins worldwide in histology laboratories, among others. Paraffin is a hydrophobic reagent that cannot be mixed with the tissues embedded in water (Alturkistani et al., 2015; Everson-Pearse, 1954), so, different protocols have been developed to change the chemical properties of the tissue to make it soluble in this resin. These protocols, of sample processing for histology purposes, can vary depending on the specifics of each sample. For example, the species from which the tissue comes, its hardness and water content, among other (Alamri et al., 2017; Buesa & Peshkov, 2009; García, 1993; Munganyinka et al., 2018; Prophet et al., 1995).

Protocols for histology processing share the same histochemical logic. The first step, in the preparation of samples for plant and animal histology, is the tissue fixation, which preserve structures as close to fresh tissue as possible (Bradbury & Meek, 1960; Eltoum et al., 2001). Once the tissue is fixed, it needs to be processed to make it soluble in the resin. Processing is divided in three stages: a gradual dehydration stage to extract all water from the tissue (Becker et al., 2012; Fernandes et al., 2009; Rylander et al., 2006; Smith et al., 2008), the clearing stage that makes it soluble in the resin (Buesa & Peshkov, 2009; Zhu et al., 2013), and the tissue infiltration stage in the resin (García, 1993; Prophet et al., 1995; Sidman et al., 2001).

After processing, tissue must be embedded in the resin, micrometric cut, stained with the required histochemical technique, dehydrated, cleared, and mounted (García, 1993; Prophet et al., 1995). Depending on the protocol, plant tissue processing time can take up to 4 days (Fhaizal et al., 2013; Sandoval, 2005), but the molecular kinetics of reactions between reagents and tissues can be accelerated with a microwave to save time (Buesa, 2007b; Leong, 2004).

Histocytotechnologist, in their work, is exposed to hazardous biological and chemical agents that can affect their health and the environment if chemical wastes are arranged incorrectly (Buesa, 2007a; Roy, 1999). Derivatives of the benzene, such as the dimethyl benzene, commonly known as xylene, that is one of the most used organic solvent in histology laboratories for processing, dewaxing and mounting steps (Buesa & Peshkov, 2009; Zhu et al., 2013).

Xylene can be absorbed by dermal, inhalational, and oral route. It can be biotransformed in the liver and excreted in urine as methylhippuric acid 18 hours after the end of an acute exposure. Prolonged or chronic exposure can cause the accumulation of xylene in muscle and fat tissue. Acute inhalation causes nose and throat irritation, and chronic exposure can decrease pulmonary function, cardiovascular and gastrointestinal effects. Oral absorption can cause renal damage, depression of the respiratory system, coma, and death. Dermal exposure is associated with urticaria, toxic eczema, dryness, erythema, and vasodilatation of the skin (Niaz et al., 2015; Rajan & Malathi, 2014). Because of all these problems, histocytotechnologist must use the appropriate personal protective equipment and always work in a fume hood when they are working with xylene and other dangerous reagents (Materials and Safety Committee, 2015; Niaz et al., 2015; Rajan & Malathi, 2014; ThermoFisher-Scientific, 2010).

Another major concern is the xylene waste disposal. Because 1 L of xylol can contaminate up to 675 L of water (Ayotamuno et al., 2006; Ossai et al., 2020), it affects soil and aquatic ecosystems, it can cause acute, sub-acute and cumulative toxicity in aquatic flora and fauna (Duan et al., 2017; Neuparth et al., 2014). Therefore, laboratories must follow the local regulations and dispose of their chemical wastes correctly to avoid environmental contamination.

Based on all the occupational health and environmental problems that xylene can cause if it is not handled correctly, there have been created xylene substitutes and some xylene-free protocols. The main limitation with xylene substitutes, such as terpenes, is their price, which goes between 1.1 to 7.4 times more expensive than xylene. For animal tissue there are alternative protocols that embed the tissues in paraffin and did not use any derivative of benzene for clearing (Buesa & Peshkov, 2009), there are no a reproducible benzene derivatives-free protocols that embed plant tissue in paraffin.

Some protocols for plant tissue, including seeds, that used glycol methacrylate and hydroxyethyl-methacrylate as embedding resin that is a hydrophilic resin that avoids the use of organic solvents for processing (Tessmer et al., 2022; Zraidi et al., 2003). The inconvenience with methacrylate resins is that are between 5 to 12.5 times more

expensive than paraffin, they need longer period for infiltration, embedding and a difficult ultra violet-dependent polymerization (Santos de Oliveira, 2015; Samavedi et al., 2014). Some hard resins also need an ultramicrotome to be able to cut the resin, instead of the common rotatory microtome of histology laboratories (Kshatriya et al., 2018).

The goal of this research was to compare two new protocols for seed histology accelerated with microwave, tested on seeds of economic importance species.

Materials and methods

Sample collection and protocol optimization were done between January and May of 2022. The seeds of *C. arabica* var. Obata, *G. max* var. CIGRAS-06, *P. vulgaris* var. Tayni, *O. sativa* var. Lazarroz FL, and *Z. mays* var. EJM-2 were requested to the Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica. The seeds of *C. papaya* var. Pococi were requested to the Instituto Nacional de Innovación y Transferencia en Tecnología Agropecuaria (INTA) of Costa Rica. The protocol optimization was done at the CIGRAS histology laboratory.

Three technical replicates of five seeds of each species were tested with the xylene-free and xylene-dependent protocols, on different days for each replicate. The batches from which the seeds were taken were made up of seeds from different plants.

All sampled seeds were soaked for 12 h. Then, they were split in half and placed on histology cassettes. The testa of *C. arabica* seed, and the lemma and palea of *O. sativa* were removed before fixing to facilitate penetration of the fixation and processing reagents. All of them were fixed in F.A.A. solution, compound by 10 % formalin at 37 % v/v, 5 % glacial acetic acid, and 85 % ethanol at 70 % v/v, for 48 h at room temperature.

After fixing, XD samples were dehydrated in a graded series of ethanol (70, 80, 90, 95, and two changes of 100 % v/v) heated in a microwave³ to reach 50-60 °C and kept cooling at room temperature for 10 min each one. After dehydration, samples were cleared in a 1:1 rate solution of 100 % ethanol: xylene and two changes of xylene, each reagent was heated in a microwave to reach 50-60 °C and kept cooling at room temperature for 10 min. Then, samples were infiltrated in a 1:1 rate solution of xylene-Paraplast® for 20 min, and in two changes in Paraplast® for 40 min, each solution was kept on a stove at 60 °C. In the last Paraplast® step samples were placed in 40 mm Hg of vacuum for 5 min to takeout possible air bubbles from tissue. XD protocol was taken from Benavides-Acevedo (2021) with minor modifications.

In the case of the BDF samples, after fixing, they were dehydrated in a graded series of isopropanol (70, 80, 90, 95, and two changes of 100 % v/v) heated in a microwave to reach 50-60 °C and kept cooling at room temperature for 10 min each. Then, samples were cleared in 5:1 and 2:1 rate solutions of isopropanol: mineral oil and in mineral oil, heated on a stove at 60 °C for 40 min each one. After clearing, samples were infiltrated in a 1:1 rate solution of mineral oil: Paraplast® for 20 min, and two changes of Paraplast® for 40 min, each solution was kept on a stove at 60 °C. In the last Paraplast® step samples were placed in 40 mm Hg of vacuum for 5 min to takeout possible air bubbles from tissue. This BDF protocol was taken from Buesa & Peshkov (2009) with major modifications.

Samples of both protocols were embedded in Paraplast plus®, sectioned in 5 µm slices with a rotatory microtome, placed in a flotation bath with water at a temperature between 40-45 °C, collected with a slide, and incubated on a stove at 60 °C for 1 h. Dewaxing of XD samples was made with two changes of xylene, and hydration with a graded series of ethanol (two changes of 100, 95 and 70 % v/v) and tap water for 2 min each one. In the case of BDF samples, dewaxing was done with two changes a solution of 2 mL of a concentrated neutral detergent (Ticolab, Costa Rica)

³ It is important that samples were immersed in each reagent and heated in the microwave together. This applies for all heating in the microwave steps of both protocols.

diluted in 98 mL of tap water, this solution was heated between 75-80 °C, and each sample was immersed into it for 2 min. Then, each sample was rinsed in two changes of tap water for 2 min (Buesa & Peshkov, 2009).

Hydrated sections of both protocols were stained with Acid Periodic of Schiff (PAS) – Coomassie Blue, which works as topographic staining, to characterize general anatomy, and as functional staining for neutral glycosaminoglycans, as starch and cellulose, with the Schiff's leukofuchsin and proteins with the Coomassie Blue. This was based on the protocol of Schmidt et al. (2012) with own modifications. For this purpose, hydrated slides were oxidated with periodic acid at 1 % w/v for 15 min and rinsed three times with deionized water for 1 min each one. Then, the slides were stained with Schiff's reagent for 15 min and rinsed with tap water for 5 min. Counterstain was done with Coomassie Blue at 0.02 % w/v solved in a solution of methanol: acetic acid: water in a 46.5:7:46.5 rate (Mochizuki & Furukawa, 1987). The Coomassie Blue solution with the slides inside was warmed in the microwave to 50 °C and kept cooling at room temperature for 10 min.

After the staining, samples of XD protocol were dehydrated with a graded series of ethanol (95 and two changes of 100 % v/v), cleared with two changes of xylene, and mounted with CV Mount (Leica, headquarters in Wetzlar). BDF samples, after staining, were mounted in Kaiser mounting medium. Embryo and endosperm or cotyledons of all samples were histologically and cytologically characterized in a Zeiss Axio microscope and photomicrographs were taken with an Olympus DP74 camera.

Results

XD processing protocol lasted 180 min while BDF lasted 240 min. BDF was longer because of the incubation of mineral oil steps on the stove (Table 1).

Table 1. Comparison of the duration between xylene-dependent and benzene derivatives-free histological processing protocols. Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica, 2022.

Cuadro 1. Comparación de la duración entre los protocolos de procesamiento histológico dependiente de xilol y libre de derivados del benceno. Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica, 2022.

Protocol	Xylene-dependent		Benzene derivatives-free		
	Stage	Reagent	Timing (min)	Reagent	Timing (min)
1. Dehydration		Ethanol 70 %	10 *	Isopropanol 70 %	10 *
		Ethanol 80 %	10 *	Isopropanol 80 %	10 *
		Ethanol 90 %	10 *	Isopropanol 90 %	10 *
		Ethanol 100 %	10 *	Isopropanol 100 %	10 *
		Ethanol 100 %	10 *	Isopropanol 100 %	10 *
2. Clearing		Ethanol: xylene (1:1)	10 *	MO: Isopropanol (5:1)	30 **
		Xylene	10 *	MO: Isopropanol (2:1)	30 **
		Xylene	10 *	MO	30 **
3. Infiltration		Xylene: Paraplast® (1:1)	20 **	MO: Paraplast® (1:1)	20 **
		Paraplast®	40 **	Paraplast®	40 **
		Paraplast®	40 **	Paraplast®	40 **
Processing time			180 min	240 min	

* Heated on a microwave between 50-60 °C and cooled at room temperature for the indicated time. / * Calentado en microondas entre 50-60 °C y enfriado a temperatura ambiente por el tiempo indicado.

** Incubated on a stove at 60 °C. / ** Incubado en una estufa a 60 °C.

The total time of dewaxing, hydrating, staining, dehydration, clearing, and mounting was 72 min for XD and 56 min for BDF protocol (Table 2).

Table 2. Comparison of the duration between the deparaffinization, hydration, staining, and histological mounting protocols dependent on xylene and benzene derivatives-free. Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica. 2022.

Cuadro 2. Comparación de la duración entre los protocolos de desparafinización, hidratación, tinción y montaje histológico dependiente de xilol y libre de derivados del benceno. Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica. 2022.

Protocol	Xylene-dependent		Xylene-free	
	Reagent	Timing (min)	Reagent	Timing (min)
1. Dewaxing	Xylene	2	Neutral detergent 2 %	2 *
	Xylene	2	Neutral detergent 2 %	2*
2. Hydrating	Ethanol 100 %	2		
	Ethanol 100 %	2	Tap water	2
	Ethanol 95 %	2	Tap water	2
	Ethanol 70 %	2		
	Tap water	2		
3. Staining	Periodic acid 1 %	15	Periodic acid 1 %	15
	Distilled water	3	Distilled water	3
	Schiff reagent	15	Schiff reagent	15
	Tap water	5	Tap water	5
	Coomassie Blue	10**	Coomassie Blue	10**
4. DCM	Ethanol 95 %	2	Mounting medium	-
	Ethanol 100 %	2		
	Ethanol 100 %	2		
	Xylene	2		
	Xylene	2		
	Mounting medium	-		
Total time		72 min		56 min

* Heated on a microwave between 75-80 °C and cooled at room temperature for the indicated time. / * Calentado en microondas entre 75-80 °C y enfriado a temperatura ambiente por el tiempo indicado.

** Incubated on a microwave to 50 °C and cooled at room temperature for the indicated time. / ** Calentado en microondas a 50 °C y enfriado a temperatura ambiente por el tiempo indicado.

DCM: dehydration, clearing and mounting. / DCM: deshidratación, aclaramiento y montaje.

In *C. papaya*'s seeds processed with the xylene-dependent protocol the embryo, endosperm, mesotesta, and integuments were well preserved and processed (Figure 1 A and C). Benzene derivative-free protocol papaya seeds were more difficult to cut, compared with xylene-dependent protocol seeds. This cutting difficulty caused some artefacts, especially in the endosperm (Figure 1 B), but, the embryo, mesotesta and integuments were well preserved (Figure 1 B and D).

Papaya's embryo had a high cell density, formed by homogeneous small cells, compared with the endosperm cells, with a prominent nucleus and visible PAS+ cytoplasmic content. Protein content was not homogeneous along the embryo, there were more proteins towards the root pole (Figure 1 A and B). Endosperm was surrounded

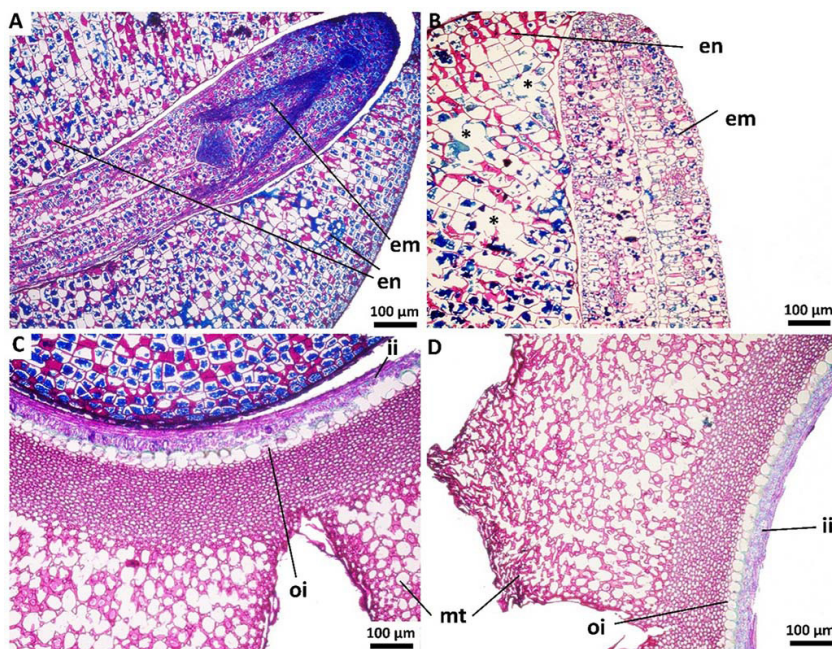


Figure 1. Photomicrographs of *Carica papaya* var. Pococí taken at 200X and stained with Periodic Acid of Schiff and Coomassie Blue. The left column is of samples processed with the xylene-dependent protocol; the right column is of samples processed with the benzene derivative-free protocol. A and B. Longitudinal section of the embryo (em) and endosperm (en). Asterisks (*) mark cutting artefacts on the endosperm. C and D. Longitudinal section of the mesotesta ridge (mt), outer integument (oi) and inner integument (ii). Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica. 2022.

Figura 1. Fotomicrografías de *Carica papaya* var. Pococí tomadas a 200X y teñidas con ácido peryódico de Schiff y azul de Coomassie. La columna de la izquierda es de muestras procesadas con el protocolo dependiente de xilol. La columna de la derecha es de muestras procesadas con el protocolo libre de derivados de benceno. A y B. Cortes longitudinales del embrión (em) y del endospermo (en). Los asteriscos (*) marcan artefactos del corte micrométrico. C y D. Cortes longitudinales de la cresta de la mesotesta (mt), el integumento externo (oi) y el integumento interno (ii). Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica, 2022.

by monolayer of the epidermis and its parenchyma was formed by heterogeneous size cells with a medium dense cytoplasm that stored carbohydrates and proteins (Figure 1 A and B). The mesotesta was a PAS+ death cell matrix, possibly formed by structural carbohydrates such as cellulose, without any protein. The outer integument was formed by medium size death cells without visible contain. The inner integument was made of small living cells that towards the endosperm were covered by a PAS+ laminar layers possible formed by cellulose (Figure 1 C and D).

C. arabica seeds kept their expected histological characteristics with both processing protocols, cells had continuous cell walls, were geometrically shaped, and had well-preserved nuclei (Figure 2). Embryo cells were smaller than the endosperm, half of them had a dense, PAS+, cytoplasmic content, almost without any visible protein present (Figure 2 A and B). Endosperm had a monolayer epidermis of polyhedral cells with some PAS+ and protein contained in their cytoplasm. Endosperm's parenchyma was formed by heterogenous shaped and sized cells with cytoplasm with a moderate PAS+ starch contain and proteins (Figure 2). In the medullar parenchyma there were found brown deposits of an unknown substance (Figure 2 C and D).

G. max seeds histological features with both protocols were as expected. Cells kept their integrity and structure (Figure 3). Soybean embryonic axis was surrounded by a monolayer of protodermis formed by small, and squared cells. At root, the poles were the root meristem and the root parenchyma. The central cylinder was surrounded by

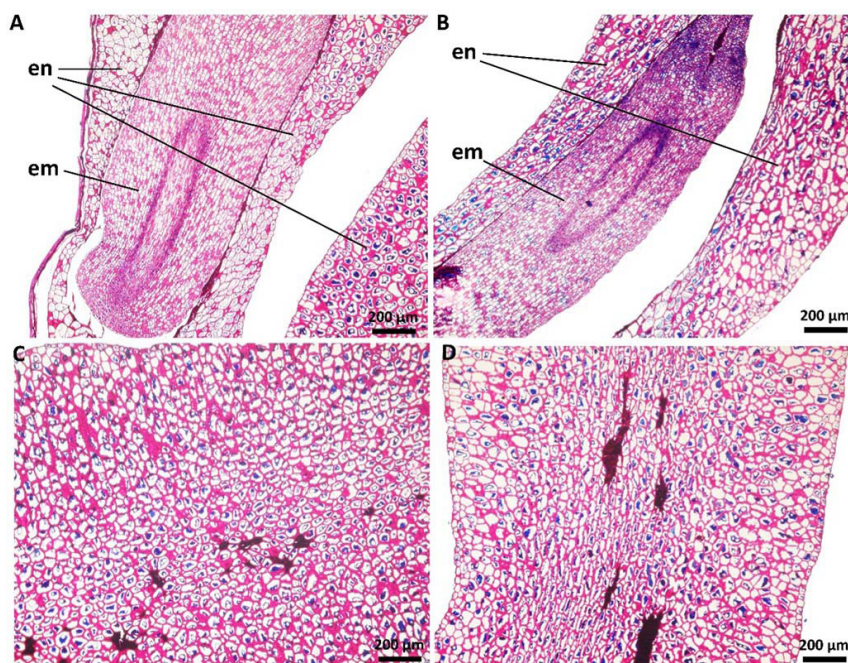


Figure 2. Photomicrographs of *Coffea arabica* var. Obata taken at 100X and stained with Periodic Acid of Schiff and Coomassie Blue. The left column is of samples processed with the xylene-dependent protocol; the right column is of samples processed with the benzene derivative free protocol. A and B. Longitudinal section of the embryo (em) and endosperm (en). C and D. Longitudinal sections of the endosperm. Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica, 2022.

Figura 2. Fotomicrografías de *Coffea arabica* var. Obata tomadas a 100X y teñidas con ácido peryódico de Schiff y azul de Coomassie. La columna de la izquierda es de muestras procesadas con el protocolo dependiente de xilol. La columna de la derecha es de muestras procesadas con el protocolo libre de derivados de benceno. A y B. Cortes longitudinales del embrión (em) y del endospermo (en). C y D. Cortes longitudinales del endospermo (en). Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica, 2022.

the vascular system of the embryo. At the apical pole was the hypocotyl (Figure 3 A and B). The cotyledons were enclosed by its monolayer of epidermis formed by cells with a dense PAS+ cytoplasm and prominent nucleus. The cortical parenchyma was compound by bigger cells that the ones from its epidermis, which had a dense proteinaceous cytoplasm that camouflages its nuclei. The medullar parenchyma was formed by cells with a variable cytoplasm contain, some cells had an empty cytoplasm, others had a light PAS+ starch content and others had a dense proteinaceous contain (Figure 3 C and D).

O. sativa seeds were well conserved and processed with both protocols. The cells of the living structures of the seeds maintained their structure and integrity (Figure 4). Rice's embryos were taken in a peripheral (Figure 4 A) and medial (Figure 4 B) section. Their cells were small, compact and had a high density, their cytoplasm was dense and highly proteinaceous with hints of PAS+ carbohydrates (Figure 4 A and B). The endosperm was formed by acellular starch deposits that showed artefact cracks and were surrounded by the aleurone and the pericarp (Figure 4 C and D).

P. vulgaris' seeds were well fixed and processed, and their structures kept the correct anatomical characteristics in samples of both protocols (Figure 5). The main structures of the embryonic embryo were the root pole with its rood meristem, root parenchyma and the protodermis surrounding it. The central cylinder was surrounded by the

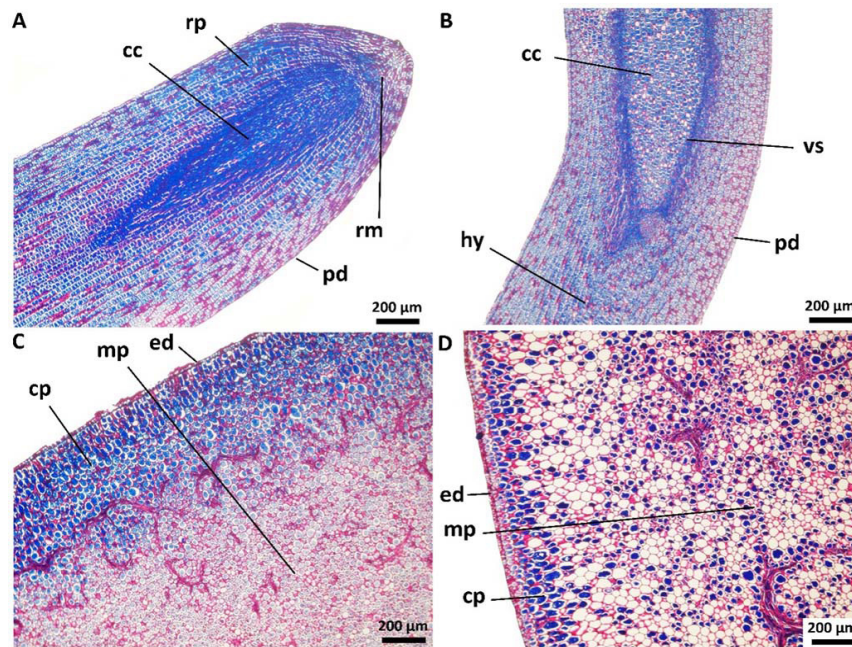


Figure 3. Photomicrographs of *Glycine max* var. CIGRAS-06 taken at 100X and stained with Periodic Acid of Schiff and Coomassie Blue. The left column is for samples processed with the xylene-dependent protocol; the right column is of samples processed with the benzene derivatives-free protocol. A and B. Longitudinal sections of the embryonic axis with its root meristem (rm), root parenchyma (rp), central cylinder (cc), vascular system (vs), hypocotyl (hy), and protodermis (pd). C and D. Longitudinal section of the cotyledon with its epidermis (ed), cortical parenchyma (cp), and medullar parenchyma (mp). Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica. 2022.

Figura 3. Fotomicrografías de *Glycine max* var. CIGRAS-06 tomadas a 100X y teñidas con ácido peryódico de Schiff y azul de Coomassie. La columna de la izquierda es de muestras procesadas con el protocolo dependiente de xilol. La columna de la derecha es de muestras procesadas con el protocolo libre de derivados de benceno. A y B. Cortes longitudinales del embrión con sus cotiledones (co), hipocótilo (hy) y protodermis (pd). C y D. Cortes longitudinales del endospermo con su epidermis (ed), parénquima cortical (cp) y parénquima medular (mp). Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica, 2022.

vascular system. At the shoot, poles were the hypocotyl and the shoot meristem (Figure 5 A and B). The cotyledons were surrounded by an epidermis formed by small, and squared shape cells with a dense proteinaceous cytoplasm that cover their nuclei. The cortical parenchyma was compounded by medium size cells, bigger than the ones of the epidermis but smaller than the ones of the medullar parenchyma, with a dense cytoplasm full of protein and carbohydrates. The medullar parenchyma's cells shared the same characteristics as the cortical parenchyma, except that their cells were bigger (Figure 5 C and D).

Z. mays' seeds were well fixed and processed with both protocols, cells of the living structures of the seeds maintain their microscopic structure (Figure 6). The basic structure of the seeds is composed of the embryo (which has a high concentration of proteins), the endosperm (with uniform cells and high content of starch) and the seed coat. These seeds were difficult to cut because of the their high starch content that made tissue breakable while cutting. The embryos were formed by a high cell density with a dense proteinaceous contain with PAS+ starch

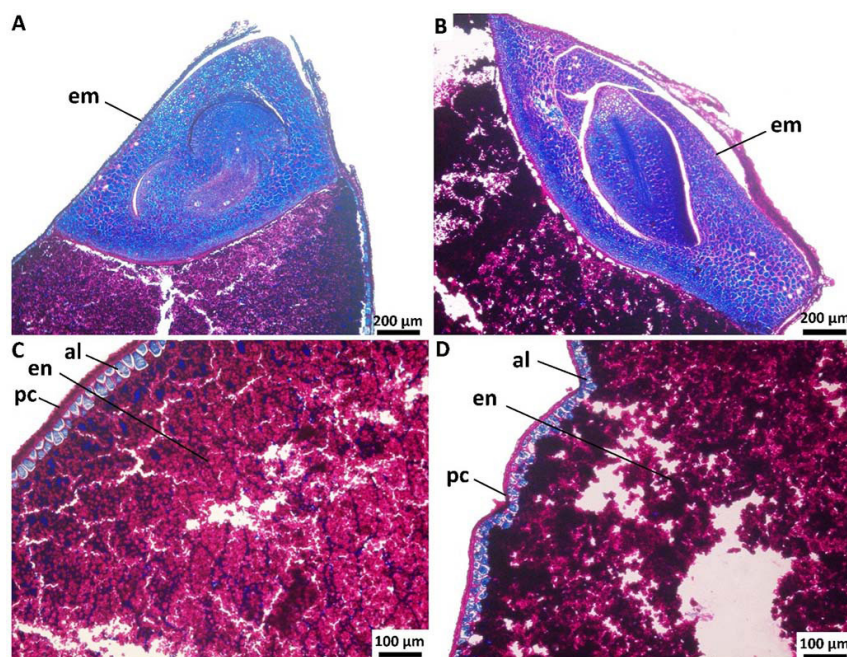


Figure 4. Photomicrographs of *Oryza sativa* var. Lazarroz FL stained with Periodic Acid of Schiff and Coomassie Blue. The left column is of samples processed with the xylene-dependent protocol; the right column is of samples processed with the benzene derivatives-free protocol. A and B. 100X longitudinal sections of the embryo. C and D. 200X longitudinal sections of the pericarp (pc), aleurone (al), and endosperm (en). Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica. 2022.

Figura 4. Fotomicrografías de *Oryza sativa* var. Lazarroz FL teñidas con ácido peryódico de Schiff y azul de Coomassie. La columna de la izquierda es de muestras procesadas con el protocolo dependiente de xilol. La columna de la derecha es de muestras procesadas con el protocolo libre de derivados de benceno. A y B. Cortes longitudinales a 100X del embrión. C y D. Cortes longitudinales a 200X del pericarpio (pc), la aleurona (al) y del endospermo (en). Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica, 2022.

deposits (Figure 6 A and B). The endosperm was an acellular matrix of starch deposits that presented some cracks and empty spaces as cutting artefacts (Figure 6 C and D).

Discussion

XD and BDF protocols allowed to obtain seed histology slides and microphotographs. The PAS-Coomassie Blue staining worked quite well as a morphological staining and as a functional one. It made a good differentiation of carbohydrates and proteins (Figure 1-6).

Both protocols have the advantage of being quick protocols that takes a total time of 4.2 h and 4.9 h, XD and BDF, respectively (Table 1 and 2). This is a great benefit compared with other protocols for plant histology that can take several days or even weeks to obtain histology slides (Fhaizal et al., 2013; Sandoval, 2005). Both protocols used the increase of molecular kinetics, caused by microwaves, to accelerate the penetration, reactions and molecular exchange between reagents and tissues (Buesa, 2007b; Leong, 2004). Another advantage of these protocols is that they are manual protocols, that can be used in any laboratory that has a microtome, a stove, a fume hood (for XD protocol), a microwave, a vacuum pump (if necessary), and basic glassware.

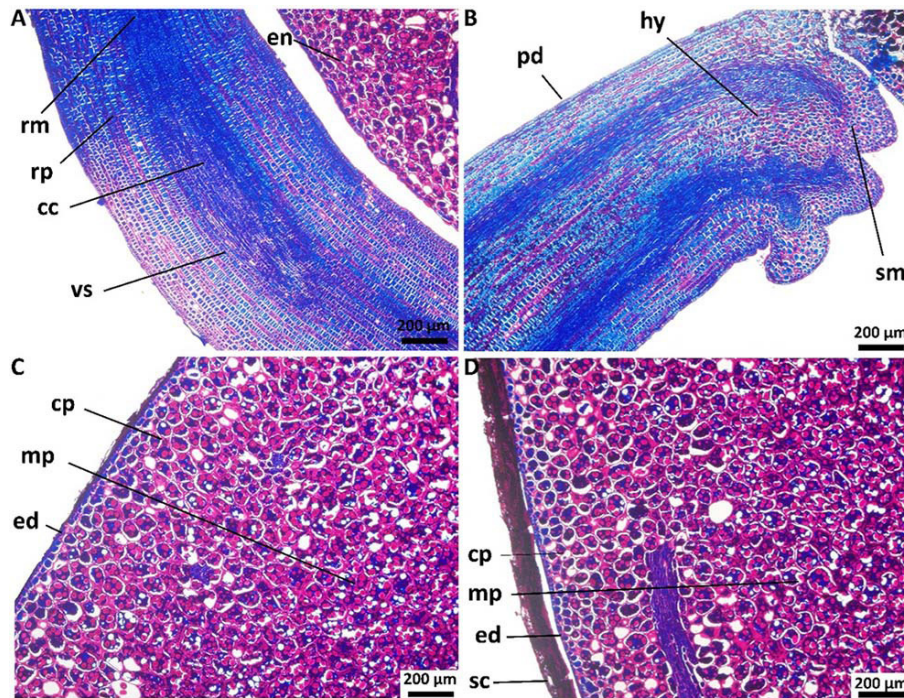


Figure 5. Photomicrographs of *Phaseolus vulgaris* var. Tayni taken at 100X and stained with Periodic Acid of Schiff and Coomassie Blue. The left column is of samples processed with the xylene-dependent protocol; the right column is of samples processed with the benzene derivative free protocol. A and B. Longitudinal sections of the embryo with its cotyledons (co) and hypocotyl (hy). C and D. Longitudinal sections of the endosperm (en) surrounded by the endodermis (ed) and formed by its cortical parenchyma (mp), and by the medullary parenchyma (mp). Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica, 2022.

Figura 5. Fotomicrografías de *Phaseolus vulgaris* var. Tayni tomadas a 100X y teñidas con ácido peryódico de Schiff y azul de Coomassie. La columna de la izquierda es de muestras procesadas con el protocolo dependiente de xilol. La columna de la derecha es de muestras procesadas con el protocolo libre de derivados de benceno. A y B. Cortes longitudinales del embrión con sus cotiledones (co) e hipocótilo (hy). C y D. Cortes longitudinales del endospermo (en) rodeado por la endodermis (ed) y formado por su parénquima cortical (cp) y por el parénquima medular (mp). Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica, 2022.

XD protocol is 44 min faster than BDF protocol. But BDF protocol has the advantage of does not use any benzene derivatives. This is very important for the environment, to reduce the dependence on petroleum and the contamination with petroleum derivatives, and for the job safety of histocytotechnologist around the world (Duan et al., 2017; Neuparth et al., 2014; Niaz et al., 2015; Ossai et al., 2020; Rajan & Malathi, 2014).

The dewaxing strategy of BDF protocol with a heated solution of a neutral detergent (Buesa & Peshkov, 2009) was as good as the common dewaxing with xylene and hydration with a gradient of alcohols. Samples of both protocols did not show any rest of Paraplast® in any photomicrographs (Figure 1-6). BDF dewaxing strategy could be implemented in any paraffin embedded sample, no matter how it was processed, to save dewaxing reagents and time.

The CV Mounting medium of XD protocol, like other xylene-soluble mounting mediums, makes a permanent mounting of the cover slide. This allows its conservation for many years. Kaiser mounting medium is a temporary mounting medium, for samples that can be observed on the same day after staining and do not need to be preserved longer (García, 1993; Prophet et al., 1995; Sandoval, 2005). This limits the application of the Kaiser mounting

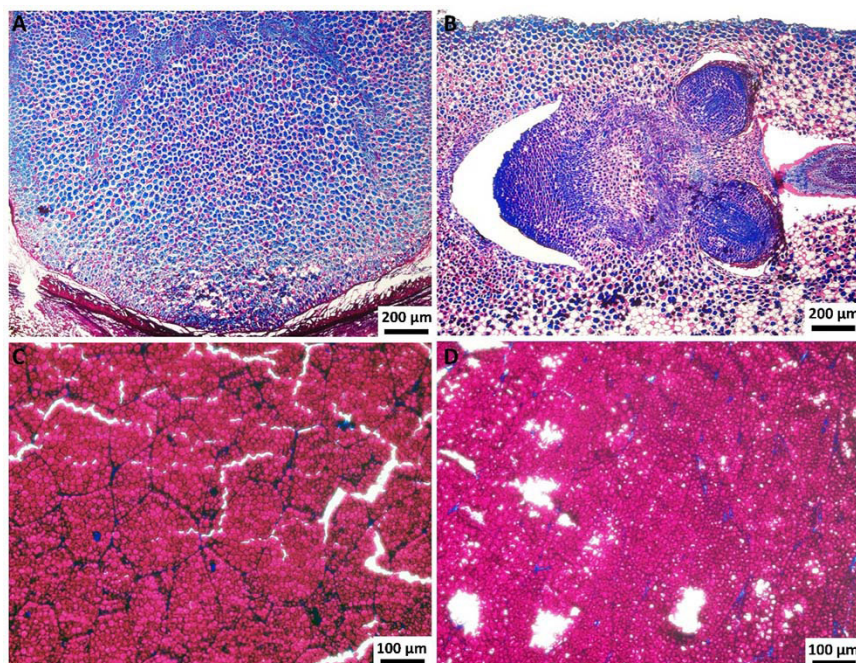


Figure 6. Photomicrographs of *Zea mays* var. EJN-2 stained with Periodic Acid-Schiff and Coomassie Blue. The left column is of samples processed with the xylene-dependent protocol; the right column is of samples processed with the benzene derivative-free protocol. A and B. 100X Longitudinal sections of the embryo. C and D. 200X Longitudinal section of the endosperm. Centro para Investigaciones en Granos y Semillas (CIGRAS) of the Universidad de Costa Rica, 2022.

Figura 6. Fotomicrográficas de *Zea mays* var. EJN-2 teñidas con ácido peryódico de Schiff y azul de Coomassie. La columna de la izquierda es de muestras procesadas con el protocolo dependiente de xilol. La columna de la derecha es de muestras procesadas con el protocolo libre de derivados de benceno. A y B. Cortes longitudinales del embrión a 100X. C y D. Cortes longitudinales del endospermo a 200X. Centro para Investigaciones en Granos y Semillas (CIGRAS) de la Universidad de Costa Rica, 2022.

medium. But, after staining BDF samples can be also permanent mounting following the dehydration, clearing, and mounting steps of XD protocol, if needed.

This research does not pretend to do a detailed histological and cytological description of the seed of the worked species, but to show general anatomical features that support the well working of XD and BDF protocols. All the described features are supported in the literature, reported for other varieties of the worked species, for bean (Recek et al., 2021; Silué et al., 2013), coffee (Amaral da Silva et al., 2004, 2008; Araujo Oliveira et al., 2020; Dedecca, 1957), maize (Liu et al., 2013; Rousseau et al., 2015; Tnani, 2012), papaya (Buides et al., 2017; Fresneda et al., 2017; Gil & Miranda, 2011; Mendes de Jesus et al., 2015), rice (Paredes et al., 2021; Ramanadane & Ponnuswamy, 2004), and soybean seed anatomy (Forti et al., 2013; Sagara et al., 2020; Shao et al., 2007; Terán, 2015; Thorne, 2015).

The artefacts are induced by one or more steps of the histology protocol. Most of the human tissue artefacts are well known (Rolls et al., 2021), but plant tissue artefacts report and descriptions are rare. Some plant tissue artefacts such as plasmolysis, cracks, shrinkage or collapse of the cell wall and vacuolar strand were reported by Feder & O'Brien (1968). The present research reported cracks in the endosperm of maize (Figure 6), papaya (Figure 1), and rice (Figure 4). They were probably induced by the high starch content of the seeds that makes tissue brittle. In the case of the brown patches in coffee's endosperm (Figure 2 C and D) is not known if it is an artefact or a substance deposit.

One of the most challenging plant tissues for histology are the seeds, because of their hardness, the presence of the seed coat, and because of the cotyledon and endosperm high starch content that makes processing and cutting

difficult. This was corroborated with the rice and maize seeds, that had a high starch contain and were the most difficult to cut on the microtome.

Based on the best of the knowledge, BDF protocol is the first protocol for plant tissue processing, in this case tested on seeds, that do not use benzene derivatives and that use paraffin as an embedding medium.

Conclusions

It is concluded that xylene-dependent (XD) and benzene derivatives-free (BDF) protocols are suitable for histological microwave-accelerated processing, of bean, coffee, maize, papaya, rice, and soybean seeds. The final slides were obtained in less than 5 h with these protocols. BDF protocol is the first for plant tissue processing that does not use benzene derivatives and that uses paraffin as an embedding medium.

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