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Seed ontogeny and endosperm chemical analysis in *Smilax polyantha* (Smilacaceae)

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Abstract. *Smilax polyantha* Grisebach is a species native to the Brazilian Cerrado biome and is known as sarsaparilla in folk medicine. Despite its popular use, little is known about the propagation of this species, which is still actively illegally exploited. The present study aims to analyse the seed ontogeny and perform endosperm chemical analyses in *S. polyantha* to elucidate the structural and chemical factors that could be associated with the low germination rates and structural organisation of the seed. The ovules are orthotropic and bitegmic, have short funicles, single collateral vascular bundles that end in the chalaza, and a hypostasis that is composed of chalazal and nucellar cells. The seed covering is non-multiplicative. In mature seeds, the cellularised endosperm has thick-walled cells, the embryo is small and the tegmen comprises two layers of periclinal elongated cells with a red–orange content, which are covered by a cuticle. Histochemical tests detected the presence of lipids, proteins and polysaccharides in the cellular content of mature seeds. Chemical analyses indicated 46.7% hemicellulose per total weight, 67.3% glucose, 30.7% mannose, 1.9% galactose and an absence of fucose, arabinose and rhamnose. In conclusion, the delayed seed germination in *S. polyantha* is associated with the seed endosperm cell walls.

Additional keywords: anatomy, mannose, sarsaparilla, seed development.

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Introduction

The Smilacaceae family consists of the *Smilax* genus, which is composed of 310 species distributed in temperate and tropical regions (Judd *et al*. 2008). In Brazil, there are 32 species distributed in several ecosystems (Andreata 2009), and *S. polyantha* is native to the Cerrado biome (savanna). This species is known as sarsaparilla and has been used in Brazilian folk medicine since the 17th century because of its anti-rheumatic properties (Moore 1895). Despite its popular use, the species is still actively illegally exploited, and few studies have addressed its propagation (Martins *et al*. 2011, 2012). These studies have shown that *S. polyantha* seeds have a lower germination rate (19–24%) that is associated with a slower speed germination index (SGI) (0.5) than for other *Smilax* species in Brazil (66–78% of germination and 1.63–4.22 of SGI). These characteristics are probably due to intrinsic metabolic factors and they are not associated with variations in temperature, substrate or light (Martins *et al*. 2012). According to Martins *et al*. (2012), no seed germination was observed after tests to break the dormancy of seeds of *S. polyantha* by using chemical scarification (sulfuric acid) and mechanical scarification (sandpaper), heat shock and gibberillic acid (GA3), following the methodology described by Santos *et al.* (2003).

From a structural perspective, there are few studies on the seeds from the *Smilax* species. The seeds are 4–8 mm in diameter, their colour varies from yellow to red, and 1–3 seeds are found per globose berry. The mature fruit exocarp colour varies depending on the species (Andreata 1997). According to Andreata (1997), the hyaline coat of the seed is elastic and originates from the ovule's outer integument; however, ontogenetic studies are needed to clarify its true origin, because there is still controversy in the field regarding this question. Sterns (1888) questioned whether the coat would be testa or aril and Dahlgren and Clifford (1982) hypothesised that the elastic structure would facilitate dispersal by birds. The endosperm is white in colour and it has a hard consistency (Andreata 1997), and *S. goyazana* is composed of thick-walled cells with lipid droplets in the vacuole (Palhares *et al*. 2009). According to the latter authors,

the embryo is dispersed at an immature stage and is positioned at the pole opposite the hilum.

Seed dormancy can be physiological when linked to chemical inhibitors, morphological when linked to embryo immaturity, or morphophysiological when the embryo is immature and there is an absence of chemical inhibitors (Ferreira and Borghetti 2004). Baskin and Baskin (2001) classified *S. glauca* and *S. rotundifolia* as having seeds with morphophysiological dormancy, on the basis of the study by Pogge and Bearce (1989). Because *S. polyantha* seeds do not exhibit difficulties in water absorption (Martins *et al.* 2011), it is likely that some structural or chemical factor in the embryo or endosperm is associated with the low rate of germination and delayed germination.

The present study aimed to evaluate the ontogeny of *S. polyantha* seeds to elucidate the origin of the elastic coat and structural factors of the seed that may be associated with the low germination rates observed for this species (Martins *et al.* 2011, 2012). In addition, we evaluated the chemical composition of the seed endosperm for the same purpose.

Materials and methods

Botanical material

The reproductive material used in the ontogenetic study of *S. polyantha* was field-collected at different developmental stages between May and September of 2006 in Pratânia, SP, Brazil. This species was identified by an expert on the genus *Smilax* in Brazil, and a voucher specimen was registered and deposited in the Herbarium collection (ESA) at the 'Luiz de Queiroz' College of Agriculture, University of São Paulo, under the number ESA 107636.

Histological analysis

The anatomical analyses were performed at different developmental stages from flower immediately after anthesis until seed maturity. For the endosperm histochemical analysis, five mature seeds were used before and soon after the onset of germination.

Three samples of each stage were fixed in a mixture of formaldehyde, glacial acetic acid and 50% ethanol (1 : 1 : 18 v/v, FAA 50) for 48 h (Johansen 1940), dehydrated in a series of graded ethanol solutions (50–100%) and embedded in plastic resin (Leica Historesin, Leica; Wetzler, Germany). The plastic blocks were sectioned into 8- to 10-μm sections. For common histological analyses, the material was stained with 0.05% toluidine blue in phosphate buffer and citric acid, with a pH between 4.0 and 6.0 (Sakai 1973) and with calcofluor white M2R to visualise the cellulose (Hughes and McCully 1975). After staining, the slides were mounted in Entellan synthetic resin (Merck, Darmstadt, Germany).

To determine the chemical nature of the substances found in the mature and developing seeds, sections from the fixed-embedded material were examined using the following histochemical tests: periodic acid–Schiff (PAS) reaction for 1,2-glycol groups present in the total polysaccharides (McManus 1948); methylene blue for mucilage (Langeron 1949); Sudan IV to identify aliphatic compounds (Jensen 1962); 10% ferric chloride to identify phenolic compounds (Johansen 1940); zinc-chloride iodine to detect starch and cell-wall material (Strasburger 1913); phloroglucin and hydrochloric acid for lignin staining (Johansen 1940); ruthenium red for pectins, polysaccharides, and acidic mucilage (Johansen 1940); and aniline blue–black for protein detection (Fisher 1968).

Standard control procedures were performed simultaneously. Photomicrographs were taken with a photomicroscope equipped with a camera. For analysis with calcofluor white, the microscope was equipped for epi-illumination with an HBO50 mercury lamp and a D filter, which provides excitation (band-pass filter: 355–425 nm) and suppression (long-pass filter: 470 nm).

Endosperm chemical analysis

The *S. polyantha* seeds were dried at 60°C, ground to a powder, and 80% ethanol was added at 80°C for 30 min to extract the soluble sugars. The sample was centrifuged, and the supernatant was separated, which was extracted with distilled water at 80°C under continuous agitation for 3 h. After the sugars were extracted, the material was filtered through a nylon-mesh matrix, and three volumes of ethanol were added to the filtrate to precipitate the hemicellulose content. The remaining material was extracted with NaOH and 4 M borohydride at 80°C under continuous agitation for 5 h. The supernatant was neutralised, dialysed and lyophilised. The fractions were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for monosaccharide analysis, after hydrolysis of the polysaccharides with 72% sulfuric acid. The oligosaccharides were analysed using thin-layer chromatography after digesting the polymers with the enzyme endo-[β]-mannanase.

Results

Histological analysis

The *S. polyantha* ovules are pendulous, orthotropic and bitegmic, and have a short funicle with collateral single vascular bundles that end in the chalaza (Fig. 1*a–d*).

The seed coat is non-multiplicative. In early seed phases, the outer integument contains 6–10 cell layers, and the inner integument is composed of two layers (Fig. 2*a*). The hypostasis is formed by chalazal and nucellar cells with lignified walls, and the endosperm exhibits free nuclei (Fig. 2*b*, *c*). During development, the endosperm becomes cellularised (Fig. 2*d*), and the seed coat begins to exhibit large cells and idioblasts that contain phenolic compounds and raphides, respectively (Fig. 2*e*). The tegmen comprises two layers of periclinal elongated cells that are covered by a cuticle and have a red–orange colour content (Fig. 2*f*), which corresponds to the coloration observed in mature seeds. At the dispersion phase, the hyaline and elastic testa separates from the tegmen and remains attached to the seed only in the chalazal region; therefore, the embryo's protective coat is formed only by the tegmen. The cellularised endosperm has thick-walled cells, even in mature seeds (Fig. 2*g*); the embryo is small and linear (Fig. 2*h*) and is positioned close to the tegument near the micropyle, which is opposite of the hilum. The haustorial cotyledon is terminal, and the plumule is laterally displaced (Fig. 2*h*). In certain sections, it is possible to see the root pole.

The histochemical tests performed on the endosperm before seed germination (Table 1) revealed that the cells produce protein

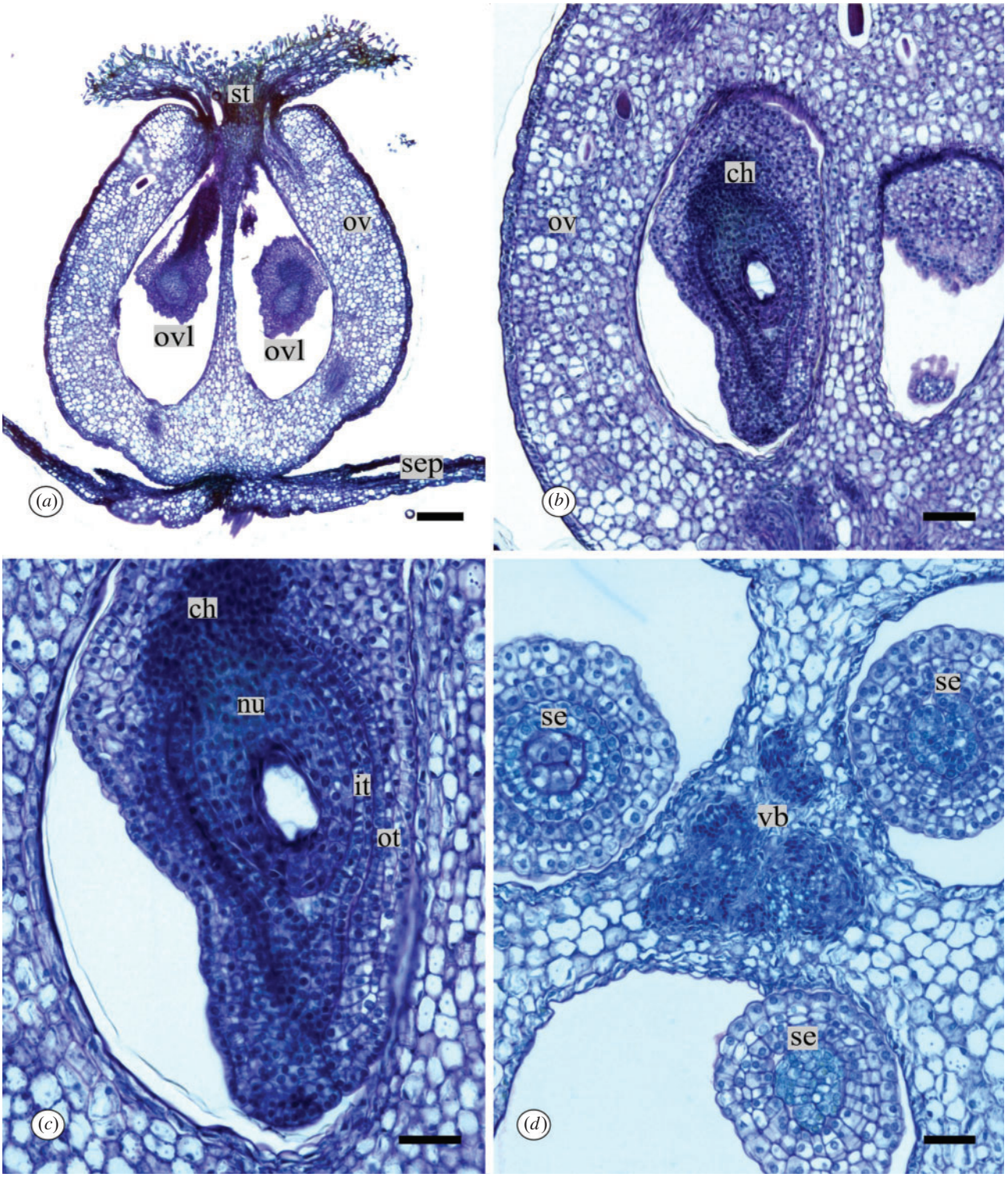

Fig. 1. (*a*–*c*) Longitudinal sections of the flower and (*d*) a cross-section of immature fruit with developing seeds of *Smilax polyantha*. ch, chalaza; it, inner integument; nu, nucellus; ot, outer integument; ov, ovary; ovl, ovule; se, seed; sep, sepal; st, stigma; and vb, ventral bundles. Scale bars = 30 µm (*c*), 50 µm (*d*), 100 µm (*b*) and 200 µm (*a*).

(Fig. 3*a*) and lipid compounds (Fig. 3*c*); however, starch is absent (Fig. 3*d*, *e*). The thickened cell wall does not contain lignin, nor acidic or basic mucilage; however, it does contain cellulose (Fig. 3*b*) and polysaccharides (Fig. 3*h*). Positive reactions with methylene blue and ruthenium red occur only in the middle lamella (Table 1).

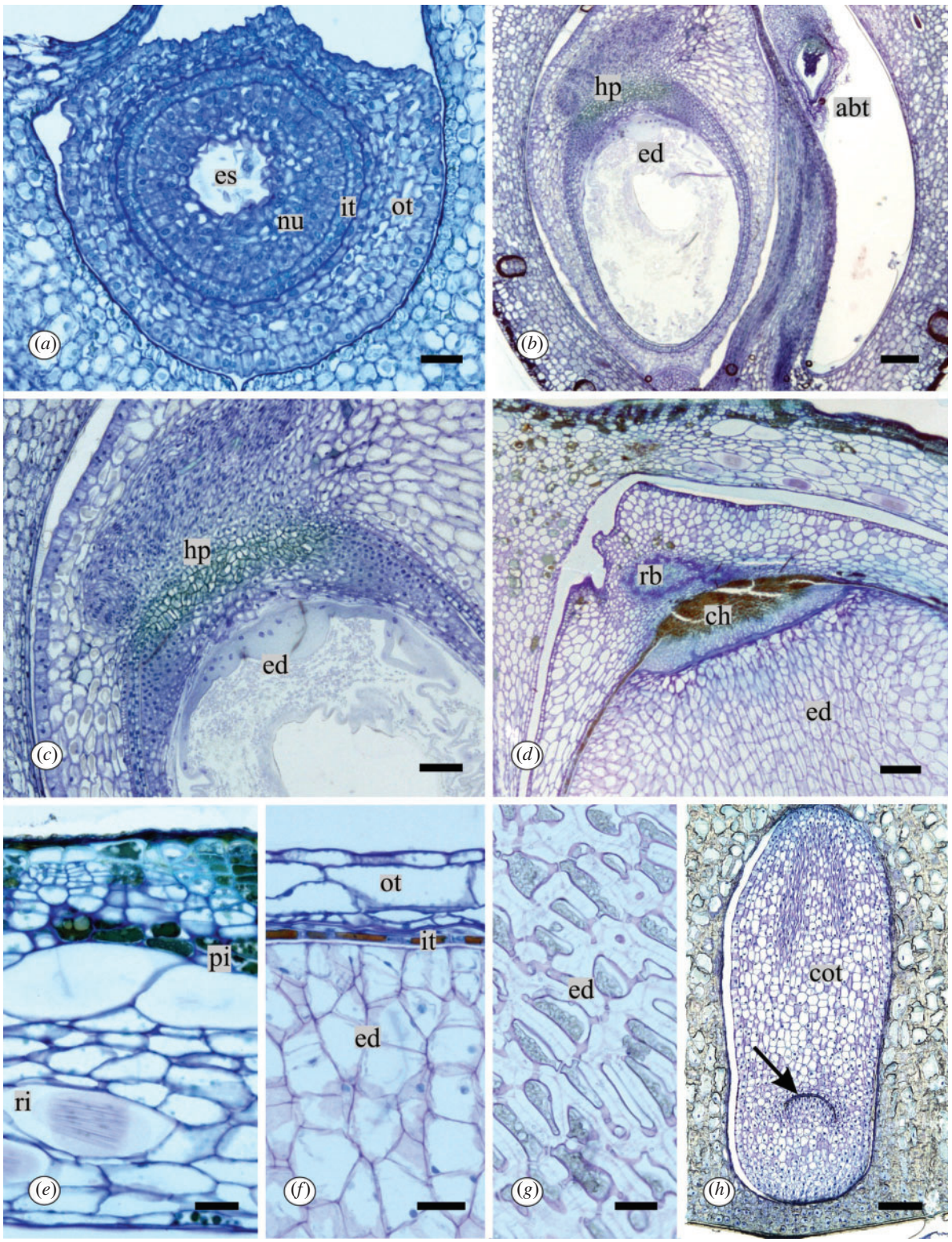

Fig. 2. Developmental stages of *Smilax polyantha* seeds. (*a*) Cross-section showing the two integuments, nucellus, and embryo sac. (*b–d*) Longitudinal sections showing (*b*, *c*) the hypostasis and endosperm in free nuclei and (*d*) cellularised phases. (*e*) Detailed image of the outer integument with phenolic idioblasts and idioblasts with raphides. (*f*) Inner integument with red–orange content and cellularised endosperm. (*g*) Detailed image of the endosperm from a mature seed. (*h*) Longitudinal section of the embryo; the arrow indicates the plumule. abt, aborted ovule; ch, chalaza; cot, cotyledon; ed, endosperm; es, embryo sac; hp, hypostase; it, inner integument; nu, nucellus; ot, outer integument; rb, rapheal bundle; and ir, idioblasts with raphides. Scale bars = 50 µm (*a*, *e–g*), 200 µm (*b*, *d*) and 100 µm (*c*, *h*).

Table 1. Histochemical tests performed on the endosperm of *Smilax polyantha* seeds
Positive signs indicate a positive reaction, and negative signs indicate the absence of reaction for the detected compounds

Staining procedure	Metabolite	Before germination		After germination	
		Cellular content reactivity	Cell-wall reactivity	Cellular content reactivity	Cell-wall reactivity
Aniline blue-black	Protein	+ (Fig. 3*a*)	–	+	–
Methylene blue	Basic mucilage	–	+	–	–
Calcofluor white		–	+ (Fig. 3*b*)	–	–
Zinc-chloride iodine	Starch and cell-wall material	– (Fig. 3*d*, *e*)	+ (Fig. 3*e*)	– (Fig. 3*f*)	– (Fig. 3*g*)
Phoroglucine (lignin)	Lignin	–	–	–	–
Reaction with periodic acid–Schiff	Polysaccharide	+ (Fig. 3*h*)	+ (Fig. 3*h*)	– (Fig. 3*i*, *j*)	– (Fig. 3*i*, *j*)
Sudan IV	Lipophilic substances	+ (Fig. 3*c*)	–	–	–
Ruthenium red	Pectins and acidic mucilage	–	+	–	–

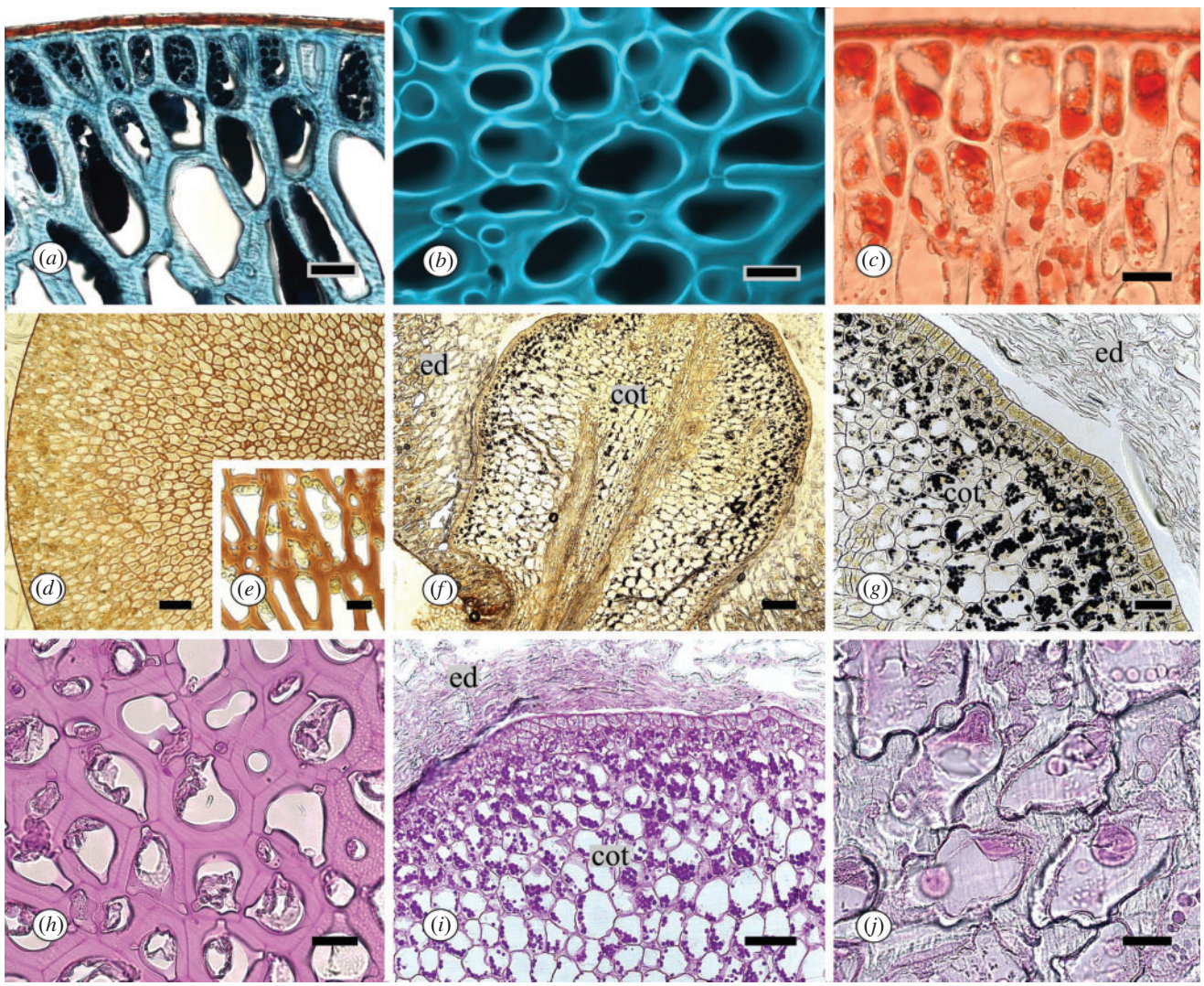

Fig. 3. Longitudinal sections of endosperm from *Smilax polyantha* seeds (*a*–*e*, *h*) before germination and (*f*, *g*, *i*, *j*) after germination. (*a*) Total protein content was visualised using aniline blue-black. (*b*) Cellulose content was visualised using calcofluor white. (*c*) Lipophilic substances were stained using Sudan IV. (*d*–*g*) Reaction with zinc-chloride iodine shows (*d*, *e*) the absence of starch granules before germination, and (*f*, *g*) the presence of starch (dark spots) in the embryo cotyledon in newly germinated seeds. (*h*–*j*) Reaction with periodic acid–Schiff shows (*h*) the thickened endosperm walls before germination, (*i*, *j*) which are degraded after germination. cot, cotyledon; and ed, endosperm. Scale bars = 30 µm (*a*–*c*, *h*, *j*), 50 µm (*e*, *g*), 100 µm (*i*) and 200 µm (*d*, *f*).

Before seed germination, there are no starch granules in the endosperm or embryo cotyledon; however, soon after germination, the emergence of starch granules in the embryo cotyledon are observed (Fig. 3*f*, *g*), and the endosperm cell walls that were previously thickened and reacted with PAS (Fig. 3*h*) are degraded (Fig. 3*i*, *j*). Proteins in the cellular content are not degraded during the germination process (Table 1).

Endosperm chemical analysis

After NaOH extraction, 46.7% of the hemicellulose per total weight was obtained from the endosperm of *S. polyantha* seeds. The percentage of the peak areas in the chromatograms for monosaccharide analysis (from digesting the cell-wall polysaccharides of *S. polyantha* with sulfuric acid) using HPAEC-PAD were as follows: 0.0% fucose, arabinose and rhamnose; 67.3% glucose; 30.7% mannose; and 1.9% galactose. The mannose-to-glucose ratio was 2.2.

Discussion

Watson and Dallwitz (1992) described Smilacaceae ovules as orthotropous, hemianatropous or campylotropous. In *S. polyantha*, the ovules are pendulous, orthotropous, bitegmic and endospermic with nuclear formation. The ontogeny of the seed coat in *S. polyantha* confirmed that the hyaline and elastic structures originated from the outer integument of the ovule, thereby corroborating the assumptions of Sterns (1888) and Andreata (1997). Therefore, the seed does not have an aril, as described by Watson and Dallwitz (1992).

According to Palhares *et al*. (2009), *S. goyazana* seeds are dispersed while the embryo is still immature and in the torpedo phase. However, in mature *S. polyantha* seeds, the embryo is well differentiated and has an expanded cotyledon and embryonic axis with shoot and root poles, which was observed in *S. quinquenervia* by Andreata and Menezes (1999) and described by Watson and Dallwitz (1992). Therefore, the low germination rate observed in *S. polyantha* (Martins *et al*. 2011, 2012) cannot be attributed to embryo immaturity, which is a type of dormancy described by Ferreira and Borghetti (2004).

In the species studied, the embryo is positioned near the seed tegument, the endosperm takes up nearly all of the space inside the seed, and the cotyledon is haustorial and develops towards the centre of the endosperm during germination, as described for *Dioscorea*, which is a genus of plants that is related to *Smilax* (Lawton and Lawton 1967). In *S. polyantha* and *S. goyazana* (Palhares *et al*. 2009), there are lipid droplets in the endosperm cells, which results in an oily endosperm (Watson and Dallwitz 1992).

In *S. polyantha*, the degradation of the endosperm wall coincides with starch accumulation in the cotyledon. The reserve polysaccharides of the cell wall occur mainly in the seeds and can be classified according to their chemical structure as mannans, xyloglucans and galactans (Buckeridge *et al*. 2000*a*). Palhares *et al*. (2009) observed a high percentage of xylose, glucose, arabinose and galactose in chemical analysis of *S. goyazana* seeds. In the present study, xylose and arabinose were not detected in *S. polyantha* seeds, the percentage of galactose was low, and glucose and mannose levels were high.

The degradation of the cell-wall reserves and starch synthesis observed in the cotyledons of *S. polyantha* may be biochemically related (Dirk *et al*. 1999) because starch is produced transiently in the cotyledons at the same time as galactomannan is degraded in the endosperm, (Buckeridge and Dietrich 1996). Horner and Arnott (1965) reported that *Yucca* seeds do not contain starch, and carbohydrates are possibly stored in the thickened endosperm cell walls. According to these authors, *Yucca* seeds before germination contain proteins and lipids in the endosperm cells, which were observed in *S. polyantha.* However, there is no protein mobilisation during *S. polyantha* germination as verified in *Yucca* seeds (Horner and Arnott 1965).

A high percentage of mannans were detected in the species studied, and mannans appear to have other functions apart from being reserve substances, such as making hard seeds to protect the embryo against mechanical damage (Buckeridge *et al*. 2000*a*).

On the basis of the monosaccharide composition of endosperm, plus the fact that the cell walls of this tissue are quite thick, looking like storage endosperm of seeds of Fabaceae, for instance (Buckeridge *et al*. 2000*a*), it can be suggested that in the case of the endosperm of *S. polyantha*, the polysaccharide present in the endosperm cell walls appear to be a galactoglucomannan, with higher percentages of glucose in the main chain. The biological function of these cell-wall polysaccharides has been thought to be related to storage (Buckeridge *et al*. 2000*b*; Buckeridge 2010) and also to the control of water entrance in seeds, a feature that seems to have adaptive value during evolution (Ferreira *et al*. 2009).

Dormancy may result from seed-coat impermeability (physical), chemical inhibition (physiological), embryo immaturity (morphological) or embryo immaturity combined with an absence of chemical inhibitors (morphophysiological) (Ferreira and Borghetti 2004). In the *S. polyantha* seeds, there was no difficulty observed for seed imbibition (Martins *et al*. 2011), seed germination was not observed after several chemical and mechanical tests to break the dormancy of seeds (Martins *et al*. 2012), and the embryo was not immature; however, there was a high amount of mannose in the endosperm walls. The mannans exhibit a high degree of intermolecular interactions and form crystals in the cell wall, which provides stiffness and reduces solubility (Buckeridge *et al*. 2000*a*). Thus, it is probable that the delayed seed germination in *S. polyantha* is associated with the seed endosperm cell walls.

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