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STRUCTURE OF *PHASEOLUS LUNATUS* TESTA AT ITS CENTRAL POINT

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In the *Phaseolus lunatus* seed, the strata and cells of the testa at the central point of the vertical side are described. The proposed regionalization and the central point technique makes it possible to define precisely each site in the testa. Exo-, meso- and endotesta are found to be basic strata in the central point studied. The exotesta is divided into cuticle, palisade and crushed cell layers. The cuticle layer is irregular and noncellular. The palisade cells are exoepidermal sclerenchyma and the crushed cells are former exoepidermal cells. The mesotesta is composed of hourglass cells, called stapes cells here because their form resembles stapes, and crushed cells. The stapes cells are parenchyma. The endotesta is composed of support tissue, three crushed cell layers, phloem, xylem and testa bottom. The endotesta adjoins the cotyledon.

Key words: *Phaseolus lunatus*, endotesta, exotesta, Malpighian cells, mesotesta, legume, testa structure.

INTRODUCTION

The testa and tegmen in a seed, together with the position of the main layer of thick-walled mechanical cells, are considered to be plant family characteristics (Corner, 1976a). Seeds are either testal or tegmic. A testal seed has a testa, which is the outer covering and may be hard. Testal seeds can be divided into exo-, meso- and endotestal seeds, according to the position of that main layer. In exotestal seeds that layer differentiates from the outer epidermis, in mesotestal seeds from the mesophyll, and in endotestal seeds from the inner epidermis (Corner, 1976a,b).

Two functions of the testa are most important: to protect the embryo in the mature seed, and to supply nutrients during seed development (Boesewinkel and Bouman, 1995). The testa has a layered structure, which strengthens its capacity to avert mechanical damage to seeds and helps prevent dehydration or possible predation (Rudall, 1987). The testa is composed mainly of cellulose and other polysaccharides, lignin, cutin, proteins, phenolic compounds, pigments, waxes, fats and resinous matter, providing the most protection against damage (Michniewicz, 1977; Bewley and Black, 1994; Werker, 1997). There is great variation in the histological structure of testas; for example, they can be very hard or fleshy, and highly sculptured (Fahn, 1974; Newell and Hymowitz, 1978; Lersten, 1979; Karcz et al., 2005).

Leguminous plants (Fabaceae) have exotestal seeds (Corner, 1976a,b). The term 'exotesta' is often used as equivalent to the outer epidermis of the (only) integument of the legume ovule, and it differentiates as a layer of radially elongated macrosclereids, which are commonly called Malpighian cells or palisade cells (Corner, 1976a, 1976b; Gunn, 1981; Werker, 1997). The testa in legumes has three structural tissue layers: the sclerenchyma, parenchyma and vasculature (Cutter, 1975). There are palisade cells (also called Malpighian cells) and vascular bundles in the testa of many legumes (Fahn, 1974). Generally, two microscopic characters distinguish the Fabaceae testa: external palisade cells and hourglass cells (Pitot, 1935a,b; Corner, 1951). Gunn (1981) reported that the testa in legumes is usually composed of seven layers: cuticle, 'light line' (absent in some legumes), epidermis (Malpighian layer), hypodermis (sclereid layer), parenchyma, remnant layers, and endosperm as the deepest testa layer. However, the testa in legumes is

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highly differentiated and its anatomical structure is species-specific (Werker, 1997).

This investigation is concerned with the testa of the lima bean (*Phaseolus lunatus* Linn.), which is a perennial American legume species with annual cultivars. It is widely distributed in Latin America, the southern United States and Canada, and many other regions worldwide. The seeds are a rich source of protein (24%) and starch (63%) (Chel-Guerrero et al., 2002). Lima beans are used for food and for the production of new materials (Betancur-Ancona, 2003). Seeds of this species are composed of the testa with the hilum, and the embryo with large cotyledons, the plumule, epicotyl, hypocotyl and radicle recognizable. The testa, in particular, is very diverse structurally and can also vary in color, according to genotype, from white to green, grey and yellow to brown, red, purple, and black (Bailey, 1963; Baudoin, 1988).

The testa of *P. lunatus* was investigated by Sterling (1954), who divided the integument (the basis for the testa) into outer stratum, middle stratum and inner stratum. He observed sclerenchyma, parenchyma, and 'mycoidal parenchyma' with a vascular system, and measured the Malpighian cells of the testa. He considered the size of testa cells to be linked to seed size. Sterling's study was related to the development of the testa but did not document its structure in the mature seed, which may differ from that in earlier stages. Although many anatomical descriptions of developing legume seed testas exist, none of them focus on their structure in the mature seed.

The testa of *P. lunatus* at the central point of the vertical side has not been investigated. The central point has not been widely accepted as a topographic marker of the seed testa. We suggest that it should be. The use of a topographic marker is important for precise regionalization of the seed testa and for comparability of testa anatomy data. This study addressed a basic question about the fully matured seed: what is the structure of the *P. lunatus* testa at the central point of its vertical side?

MATERIALS AND METHODS

PLANT MATERIAL AND SEED BIOMETRICS

White seeds of *Phaseolus lunatus* genotype AC 2441 (Fig.1), harvested in 2001, were obtained from the Institute of Applied Botany of the University of Hamburg (Germany) and stored at room temperature in the Department of Biology of the University of Joensuu. The seeds were weighed with Scaltec SBC 61 (d = 0.001 g) scales and measured using a digimatic micrometer with an electronic readout accurate to 10 μm. Seed length was measured as the longest distance along the seed, breadth as the longest distance perpendicular to length, and thickness as the depth of the seed at its thickest point (Fig. 1).

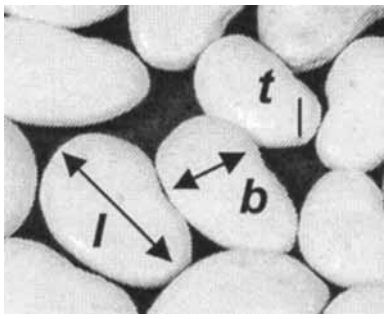

Fig. 1. Seeds of *Phaseolus lunatus*, genotype AC 2441. Measurements are indicated by arrows (I, b) and the vertical line (t). I – seed length; b – seed breadth; t – seed thickness.

ANATOMICAL STUDIES

Seed testa thickness at the central point of its vertical side was determined and measured (Fig. 2a). Cell and tissue sizes were determined by measuring cells vertically and horizontally to an accuracy of 1 μm with a micrometer. The cells were measured at their broadest in section.

Pieces of white testa were cut from the central point of the vertical side under a stereomicroscope (Stemmi 1000, Zeiss) using a scalpel and tweezers. Pieces of testa were taken from dry seeds and from seeds sandwiched in wet filter paper for 24 h.

Tissues were fixed in 2% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, rinsed after fixation, postfixed for 3 h in 1% (v/v) osmium tetroxide in 0.1 M sodium cacodylate buffer, and rinsed in sodium cacodylate buffer. After dehydration, pieces of tissue were embedded in Epon resin prepared by the standard method. For light microscopy, semithin (2 μm) sections were cut with an LKB 2188 ultramicrotome (LKB Ultrascan XL, Bromma, Sweden) and stained with toluidine blue (1%). For electron microscopy, ultra-thin sections were cut with a diamond knife and contrasted with uranyl acetate (2% w/v) and lead citrate (1% w/v, pH 12.0).

Testa samples were examined using light (Dialux 20 EB, Leitz) or electron microscopes (TEM EM 900 with MegaVision 2003). The light microscope was connected to a camera (Olympus dpi 10) and computer system (Olympus C21 W95E). The resulting images were saved and managed with MicrosoftPhotoEditor. The electron microscope images were read with MegaVision 2003 and Micrograph Picture Publisher.

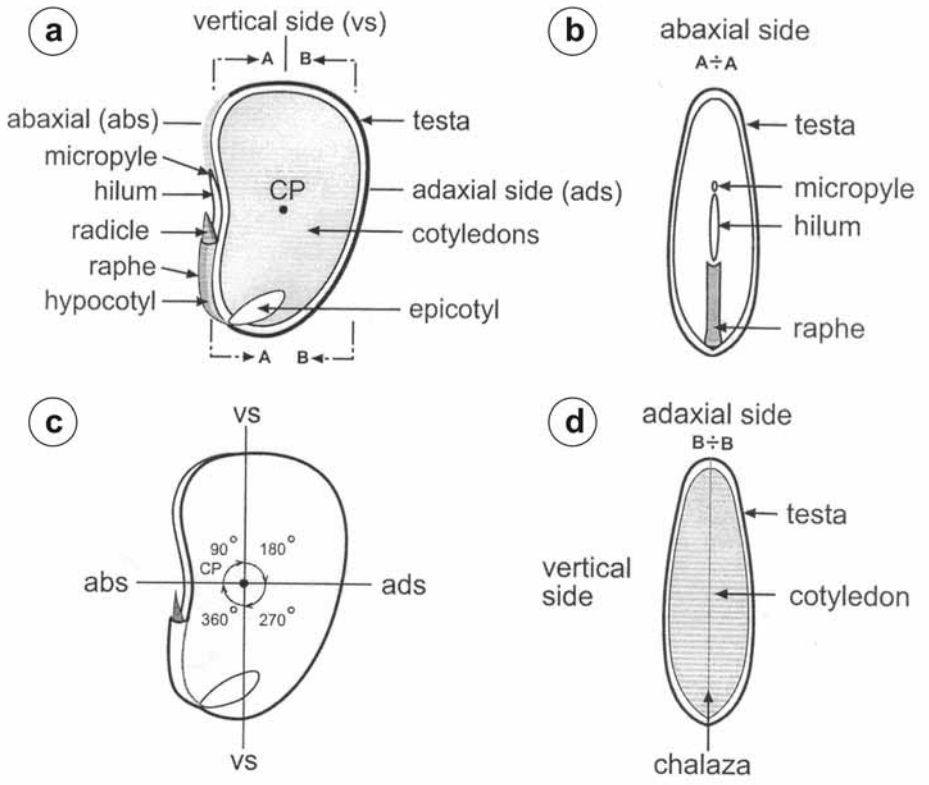

Fig. 2. Regionalization of the testa of *Phaseolus lunatus*. (**a**) Vertical side with central point and seed parts, (**b**) Abaxial side with seed parts, (**c**) Vertical side with testa regions indicated by the angle method, (**d**) Adaxial side with chalaza. vs – vertical side; abs – abaxial side; A – abaxial side direction; A÷A – abaxial side view; ads – adaxial side; B – adaxial side orientation; B÷B – adaxial side view; CP – central point.

STATISTICAL ANALYSIS

Data from measurements was analyzed using SigmaStat and SigmaPlot Version 5.0, and tested for normality at $p = 0.05$. The theoretical basis of the test for normality is that the data of a normal seed population follows a standard, bell-shaped Gaussian distribution (for details see: Aniszewski et al., 2001).

RESULTS

In our study, the central point (CP) of the testa on its vertical side was determined as the intersection of the longest vertical line dividing the seed across its breadth with the horizontal line from the middle point of the hilum (Fig. 2a). The angle of the lines at the intersection is 90°. The location of the CP was determined as 0° (360°) and 180° in the abs-ads direction, and 90° and 270° in the abs-ads direction (Fig. 2c). Figure 2b shows the micropyle, hilum and raphe at the abaxial side of the testa. The chalaza joins together the vertical sides of the testa, at the adaxial side (Fig. 2d).

The parameters of dry seeds and testas varied according to their size (Tab. 1). The median weight of seeds was 1470 mg; the median weight of testa was 15.1 mg, which was 1% of seed dry mass (Tab. 1). The median ratio of seed length/breath was 1.78, the length/thickness ratio was 4.14, and the breath/thickness ratio was 2.38; the testa/seed weight ratio was 0.0103 (Tab. 1).

The testa of a fully ripened seed of *P. lunatus* is composed of many different layers, which can be divided into three strata according to their position and the depth: the exotesta stratum (ext), mesotesta stratum (mt) and endotesta stratum (ent) (Fig. 3). Evidence of these three testa strata is presented in Figures 4–6. There are many small similar layers of crushed cells (cc) in the strata of the testa, the outermost being cc_1, the innermost cc_5 (Fig. 3).

STRUCTURE OF THE EXOTESTA

The exotesta of the mature seed is composed of a cuticle layer (c), sclerenchyma containing a palisade cell layer (p), and crushed cells (cc_1) (Fig. 4a,c). The cuticle is a very thin (<1 μm), non-regular and non-

TABLE 1. Size and weight parameters of *Phaseolus lunatus* seeds and testas

Parameter measured	Size median mm (min–max)	Weight median mg (min–max)	Coefficient
Seed weight (sw)		1470 (654–2010)	
Seed length (l)	24.83 (19.02–31.7)		
Seed breadth (b)	14.41 (12.07–18.9)		
Seed thickness (t)	6.05 (5.01–7.11)		
Testa weight (tw)		15.1 (9.0–26.0)	
l/b			1.78
l/t			4.14
b/t			2.38
tw/sw			0.0103

N = 100 seeds for sw, l, b and t; N = 10 seeds for tw.

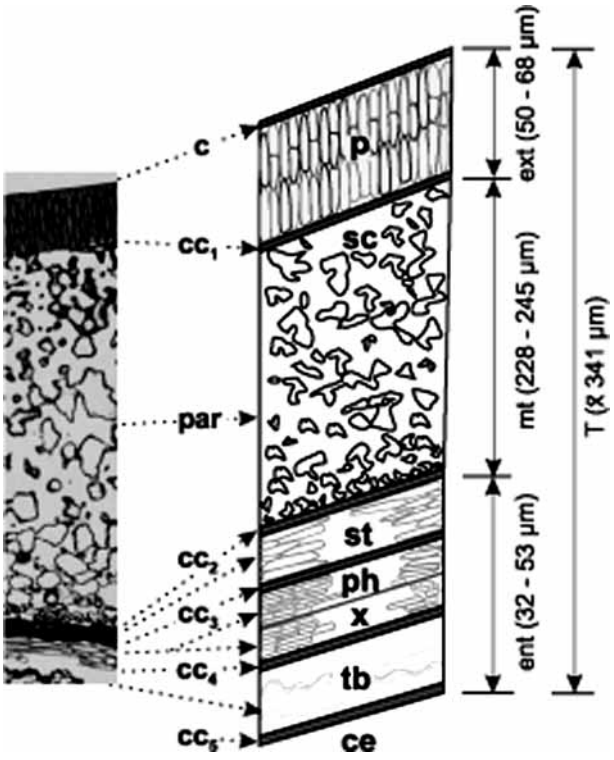

Fig. 3. Structure of *Phaseolus lunatus* testa (T) at the central point of the vertical side. At left is the light microscopy image. Measured thickness of testa (ext + mt + ent) at the central point of the vertical side was 341 μm on average. ent – endotesta; mt – mesotesta; ext – exotesta; c – cuticle layer; p – palisade layer; cc_1 – crushed cell layer in ext; par – parenchyma; sc – stapes cells; cc_2 – crushed cell layer in mt; st – support tissue; cc_3 – crushed cell layer in ent: ph – phloem; x – xylem; cc_4 – crushed cell layer in tb; tb – testa base; cc_5 – deep-base crushed cell layer; ce – cotyledon epidermis.

cellular waxy layer (Figs. 3, 4a; Tab. 2) that covers the cell walls and the spaces between cells in the external parts of the palisade layer. This waxy layer on the testa surface looks shiny to the naked eye.

The palisade layer tissue is of exoepidermal sclerenchyma, constructed from relatively large sclereids, which are palisade in form, relatively long, and pointed or not pointed (Fig. 3 at left, Fig. 4a). The drawing is a diagram of the testa structure observed by TEM, and the photomicrograph shows the structure visible by light microscopy. The two classes of cell height are presented schematically in order to visualize the structure of the testa, and are not shown to scale.

This tissue consists of thick-walled macrosclereids, which according to the literature are generally hard and lignified. The p sclerenchyma is 50–68 µm thick. The macrosclereids are 25–50 µm long and 6–16 µm wide (Tab. 1). The p tissue is at least 2–3 sclerotic cells deep (Fig. 3 at left) and comprises 15–17% of the total testa thickness. These sclerotic cells are also observed by TEM, which means that the layered structure of the macrosclereids is not an artefact of diagonal sectioning. The macro-sclereids in the palisade layer are connected together in a stable arrangement (Fig. 4b).

The p sclereids are connected to a very thin layer of crushed cells (cc_1) or an adhering layer between the ext and mt (Figs. 3, 4c). Its thickness varies but generally is <1 µm (Tab. 2).

STRUCTURE OF THE MESOTESTA

The mesotesta is composed of two different layers: thin-walled parenchyma cells (par) with a combination of other cell types, and a layer of crushed cells (cc_2) (Figs. 3, 4d,e). The mt accounts for 67–72% of the testa's total thickness in the region studied, and is composed of thin-walled parenchyma cells. The outer layer of par is clearly visible; its cells are large, semicircular, hourglass- or stirrup-shaped, but elongated (stapes cells, sc). These cells have a thick wall and are attached to the cc_1 layer of the ext by a stapes-like structure (Fig. 4d). The sc are 20–52 μm thick and 20–40 µm broad (Table 2). Both stapes cells and parenchyma are parenchymatous cells differing in their mode of collapsing: the stapes cells cannot collapse where they adjoin the exotesta cells, while the other parenchymatous cells collapse uniformly, at any side. Between these cells are air-filled intercellular spaces. In hydrated seeds these spaces are filled with water and vesicles (Fig. 4f). The par tissue is located on a very thin layer of crushed cells (cc_2) which connect the par, sc, mt and ent (Fig. 4e).

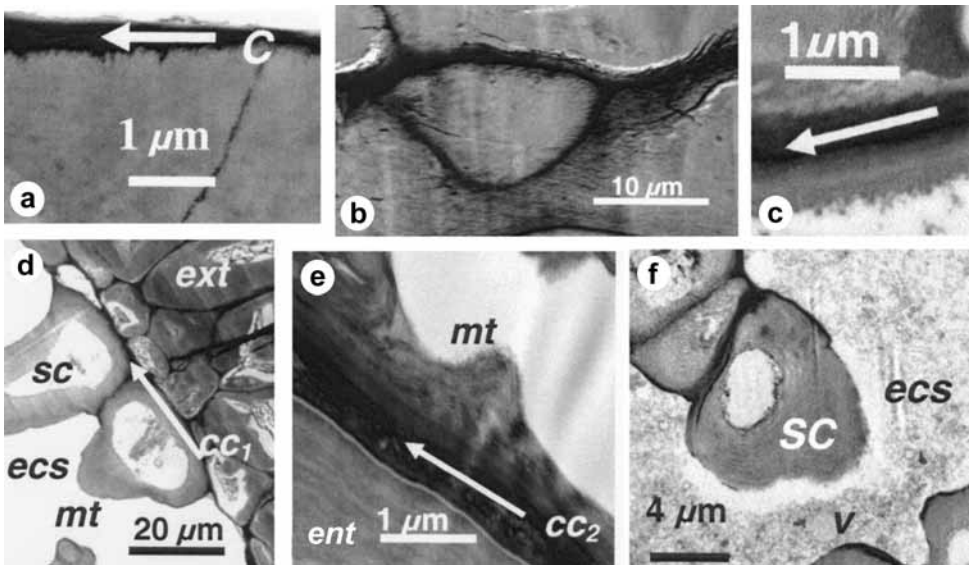

Fig. 4. Structure of *Phaseolus lunatus* testa (T) at the central point of the vertical side. (**a**) Cuticle layer (arrow, *c*) at surface of palisade cells (TEM), (**b**) Adhering structure of p cells (TEM), (**c**) Crushed cell layer cc_1 (arrow) (TEM), (**d**) Exo- and mesotesta. Sc – stapes cells; ecs – intercellular space; ext – exotesta; mt – mesotesta (TEM), (**e**) Crushed cell layer cc_2 between mt and ent (arrow). mt – mesotesta; ent – endotesta (TEM), (**f**) Mobilized cellular material in extracellular space in a soaked seed. Vesicles in extracellular spaces visible. sc – stapes cell; v – cell vesicles (TEM).

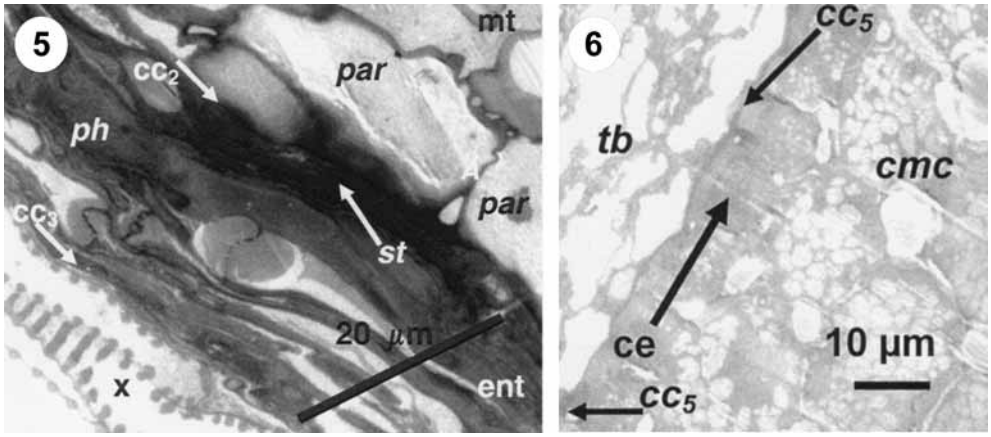

Fig. 5. Meso- and endotesta. mt – mesotesta; ent – endotesta; par – parenchymal cell; cc_2 – mt crushed cell layer; st – support tissue of endotesta; ph – phloem; cc_3 – ent crushed cell layer; x – xylem (TEM). **Fig. 6.** Boundary between testa and cotyledon. tb – testa base; ce – cotyledon epidermis (arrow); cc_5 – deep tb crushed cells; cmc – cotyledon mesophyll cells (TEM).

STRUCTURE OF THE ENDOTESTA

The endotesta is separated from the mesotesta by the crushed cell layer cc_2 (Fig. 4e). It is composed of sclerenchyma with fiber bundles and some parenchymal cells (Fig. 5), and the ent base (Fig. 6). The ent in fully matured seeds has a similar tissue structure, clearly differing from both the ext and mt (Figs. 3–6). The following layers are present in mt: support tissues (st), surface crushed cells (cc_3), phloem (ph), xylem layer (x), base crushed cells (cc_4), ent base layer (tb) and deep-base crushed cells (cc_5) (Figs. 3, 5, 6).

The base layer is 13–20 µm thick and constitutes the deepest part of the testa and its vascular system. It is composed of branched parenchyma and a cc_5 layer adjacent to the cotyledon epidermis (ce) (Fig. 6).

DISCUSSION

Our study examined the regionalization of the testa and investigated it at the central point of the vertical side. From this central point, each place in the testa can be precisely delimited. This is important for future studies of the testa, because it can vary in

TABLE 2. Measured parameters of testa strata and cotyledon thickness at the central point of the vertical side of *Phaseolus lunatus* seed

Tissue/Cell	Size (μm)	
	Thickness (min–max)	Width (min–max)
Cuticle layer (C)	<1	
Palisade layer (P)	50–68	
Palisade cells (P cells)	25–50	6–16
Ext crushed cell layer (cc_1)	<1	
Parenchyma cell layer (par)	228–245	
Stapes cells (sc)	20–52	20–40
Mt crushed cell layer (cc_2)	<1	
Support tissue (st)	7–10	
Ent crushed cell layer (cc_3)	<1	
Phloem (ph) + xylem (x)	10–19	
Crushed cell layer in testa base (cc_4)	<1	
Testa base (tb)	13–20	
Crushed cell layer in deep testa base on cotyledon (cc_5)	<1	
Cotyledon (cot)	2.7 mm	

N = 10, size to nearest 1 μm, real testa thickness = 341 μm.

structure with respect to its position on the seed (Ren and Bewley, 1998). The use of the central point and angle system as a research technique makes it possible to find identical positions in testas despite the fluctuation of seed weight and size, which is inherited (Aniszewski et al., 2001).

TESTA STRATA AND CRUSHED CELLS

There are no previous reports on the stratification of the testa of *Phaseolus lunatus* at the central point of the vertical side. Pitot (1935a,b), however, considered that the testa of leguminous plants contains external palisade and hourglass cells, and Corner (1951) mentioned the cuticle, the palisade (Malpighian cells, also called macrosclereids), hypodermal hourglass cells, the mesophyll, inner epidermal hourglass cells and vascular supply. Williams (1950) and Corner (1951) mentioned three parts of the testa: the epidermal layer of palisade cells (macrosclereids), subepidermal layer (osteosclereids with parenchyma) and aleurone layer. Many authors consider the aleurone layer in soybean to be derived from the endosperm of the ovular integument (Williams, 1950; Thorne, 1981; Baker et al., 1987; Carlson and Lestern, 1987; Yaklich et al., 1992). Thorne (1981) and Baker et al. (1987) stated that the aleurone layer of the testa is an endothelium of maternal origin. The origin of the innermost part of the testa is perhaps the most disputed area in the literature, but recent studies have provided evidence that the innermost part of the testa is actually the outermost layer of the endosperm of the ovular integument (Miller et al., 1999). Sterling (1954) divided the integument (the basis for the testa) of *P. lunatus* into the outer stratum, middle stratum and inner stratum. He observed sclerenchyma, parenchyma, and 'mycoidal parenchyma' in the testa. Sterling's strata correspond to the outer and inner epidermis and the outer endosperm of the ovular integument. Sterling's findings can also be interpreted as exo-, meso- and endotesta. In this study we documented the presence of three strata with substrata in the testa of *P. lunatus* at the studied site. We also established by TEM technique (Figs. 3–6) that the three strata show two different systems of construction: substrata constructed by cells and substrata constructed by crushed (former) cells. How crushed cells are established is not fully known, but they are probably the outcome of vigorous cotyledonal and sclereidal growth during the final stage of seed maturation when the process of cell elongation and lignification takes place. The phenomenon of flattened, degraded and crushed cells in the testa has been recognized in some other species (Ren and Bewley, 1998; Kopcińska et al., 2004). In soybean, compressed parenchyma and compressed endosperm layers in the inner surface of the testa were recently reported (Ma et al., 2004). The five crushed cell layers (cc_1–cc_5) located in the exotesta, mesotesta and endotesta found in this study suggest that crushed cells are important parts of the testa structure. The possible significance of these cells is in the protective function and energy balance of the testa during its maturation. In dead cells less food is available for potential herbivores; this also means less infection in the seed. The obliterated cells (probably nothing more than their walls) are the end-product – too costly to degrade – of the cell death that occurs during maturation of the seed testa.

PARAMETERS OF MALPIGHIAN CELLS

The presence of Malpighian (palisade) cells in the testa of leguminous plants has been widely recognized by previous investigators (Pitot, 1935a,b; Corner, 1951; Fahn, 1974; Gunn, 1981; Werker, 1997). Most cells measure between 1 μm and 100 μm (Campbell et al., 1999). In this study, the Malpighian cells were 25–50 μm thick but only 6–16 μm broad. Sterling (1954) reported that the size of testa cells is linked to seed size, and that the average length of palisade cells (Malpighian cells) of *P. lunatus* was as much as 30–60 μm, with a maximum length of even 140 μm. These macrosclereids are oriented perpen-

dicular to the cuticle layer and seed surface (Comer, 1951). In contrast to the palisade layer of the soybean testa, which is composed of a single cell layer of thick-walled macrosclereids (Miller et al., 1999), we found that exotesta macroscleroid tissue in *P. lunatus* at the adaxial side is constructed of 2–3 layers of cells. The layered structure of the macrosclereids could conceivably have been an artefact of diagonal sectioning, but this possibility was ruled out by TEM observations. The stable arrangement of the macrosclereids and tight construction of the exotesta is connected with the protective function of the testa. This adds indirect support for the presence of more than one layer of cells. Therefore, the difference from soybean in this regard can be explained by both species and seed size, as Sterling (1954) observed.

PARAMETERS OF HOURGLASS CELLS

Corner (1951) characterized hourglass cells in leguminous plants as tall, resembling pillared crypts, and intermediate in position and form between palisade-celled and stellate-celled mesophyll. Some hourglass cells have also been reported in developing seeds of such legumes as soybean and pea (Miller et al., 1999; Ma et al., 2004). In this study, the hourglass cells in the mesotesta of fully matured seeds of *P. lunatus* were found as cells in stapes form (stapes cells, sc). This name can be broadly used as a reference point for this kind of hourglass cell. Measurements of the parameters (length, width) of these cells have not appeared in the literature. They were difficult to measure because of their form and because sometimes not all of the cell was seen in the microscope image.

ENDOTESTA STRUCTURE

The endotesta is not mentioned as a recognizable layer in the mature seed testa of legumes in some general descriptive works on anatomy (Gunn, 1981; Werker, 1997). In this investigation we identified the endotesta of *P. lunatus* in the studied region as the third stratum with crushed cells, vascular and other substrata. We demonstrated its existence by TEM and presented an image of the testa base (Fig. 6). This finding is not in contradiction to studies on testa development in legumes (Williams, 1950; Corner, 1951; Sterling 1954; Thorn, 1981; Carlson and Lersten, 1987; Baker et al., 1987; Yaklich et al., 1986, 1992, 1995; Miller, 1999;). We suggest that the existence of the endotesta as a stratum is a logical consequence of the development and maturation of the testa. The vascular cell system is highly developed, with its support tissue, testa base and cc_5 layer of crushed cells. It is potentially connected with the function of the endotesta, that is, water and nutrient transport. The vascular system in legumes was observed in the 19th century and, in the case of the lima bean, was described by Sterling (1954) as two branches of bundles. The vascular system varies considerably according to the genus and/or tribe (Corner, 1951). A recent study on the enzymatic activity of testa strata (Aniszewski, 2005) has shown that the vascular system is an integral part of the endotesta, and that the enzymes SDH and AcP are active in both the endotesta and mesotesta of *P. lunatus*, strongly suggesting the presence of living cells.

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