Chapter 10

Anatomical and Chemical Insights into the White Clover (*Trifolium repens* L.) Seed Coat Associated to Water Permeability

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Abstract

White clover (*Trifolium repens* L.) seeds can exhibit physical dormancy, which produces hard seeds within a seed lot. These seeds do not germinate because they do not imbibe water due to a barrier to water entry in the seed coat. The aim of this work was to analyze the anatomical and chemical characteristics of the testa of white clover seeds with respect to water permeability levels. Seeds of the cv. NK Churrinche (2004 and 2006 harvests) were characterized via anatomical studies and determination of structural substances, polyphenols, tannins, and cutins present in the testa of seeds of different water permeability levels. Anatomically, increased testa thickness was associated with a decreased permeability level. Very slow-hydration seed coats exhibit thicker cuticle, longer macrosclereids, thicker cell wall, and the presence of wide osteosclereids than rapid-hydration seed coats; these differences are associated with a slower hydration speed and with a barrier to water entry to the seed. From the physiological and chemical points of view, the mechanism of physical dormancy of the testa would be explained by a greater amount of hydrophobic components that cement the cell wall, such as polyphenols, lignins, condensed tannins, pectic substances, and a higher proportion of cellulose and hemicellulose.

Keywords: hard seeds, testa, macrosclereids, cell wall, lignin, tannins

1. Introduction

The seed coat is the primary defense against an adverse medium, and its characteristics determine seed permeability. A hard seed coat protects the seed from mechanical stress, microorganisms, and changes in temperature and humidity [1]. There are several types of dormancy, with most of them being induced by several factors. Legume seeds have a seed coat known

as testa, which is characterized by having several layers. Outermost is the epidermis, which is uniseriate and consists of palisade macrosclereids with uneven thickened walls and interior lumen, closely packed and containing different chemical substances (quinones); the hilar region consists of two palisade layers. Below the epidermis is the hypodermis or columnar cells (osteosclereids), followed by the lacunose parenchyma, which is composed of several layers of flattened cells, the aleurone layer and the endosperm, and then the embryo [2–4].

In some leguminous species, the cuticle wax present in the hard coat plays an important role in water permeability. Some studies suggested that the osteosclereids pose the main barrier to water entry, since most seeds start to imbibe water only after those cells are perforated [3, 5]. Studies of the structure and chemical components of the seed coat of some species indicated the presence of ions, such as K, Ca, and Mg, and some phenolic compounds with a role in hardening and protection [1, 6]. The anatomy of the seed coat does not vary between hard and a non-hard seeds [7, 8]; however, some differences in content of different components, porosity, and "linea lucida" or light line were found in soybean [9]. [10, 11] related lignin content to resistance to mechanical damage in soybean cultivars; however, they exhibit hydrophobic traits and can be related to impermeability [12]. Reports on extended seed longevity are mainly related to Fabaceae and Malvaceae taxa containing Malpighian cells or osteosclereids [2, 5, 8, 13, 14]. [15] assume that dormancy breakage in these seeds (except for mechanical scarification) is due to the formation of an opening in the specialized anatomical structure of the seed coat (or of the fruit) through which water enters and hydrates the embryo.

Seed coat of white clover (*Trifolium repens*) was found to have different water permeability levels, with the corresponding different dormancy levels, which influence seed physiological quality [16]. The factors hindering water entry are not clearly defined and may be more than one. A comparative study of testas of seeds with different water permeability levels may help to explain the different dormancy levels and to determine the necessary techniques useful for breaking dormancy in seed lots. The aim of this work was to analyze the anatomical and chemical characteristics of the testa of white clover cv. NK Churrinche relative to water permeability levels.

2. Materials and methods

2.1. Seeds

Seeds of white clover cv. NK Churrinche (2004 and 2006 harvests) obtained from Criadero Barenbrug Palaversich S.A., Argentina, were used for the study. Seeds were checked under magnifying glass (10×) for purity, and those with visible physical damage were discarded.

2.2. Morphological and anatomical analysis of the testa by scanning electron microscope (SEM)

Seeds were selected according to their water permeability level; for this, seeds were classified by the hydration rate into rapid, slow, and very slow hydration [16, 17]. Seeds that imbibed water within the first 15 min were discarded due to possible damage in the seed coat. Seeds

that hydrated after 2 h of being immersed in water were classified as of rapid hydration; seeds that hydrated between 4 and 14 h of water imbibition were classified as of slow hydration; and seeds with dormancy (hard seeds) were those did not imbibe water after 22 h of immersion and were determined as of very low hydration [18]. Testas of hard seeds that were immersed in water and failed to hydrate between 3000 h (125 days) and 7488 h (312 days) were observed. For each permeability level, five testas per sample were analyzed by SEM (2004 and 2006 harvests). The testa was observed and photographed on surface and lateral views under different magnifications. In micrographs, the testa topography and cuticle appearance were observed and compared among seeds; cell layers, cell size, and cuticle thickness were determined in lateral sections. Observations were made near and around the central point [19] and in the lens area. In addition, the cell wall of macrosclereids was observed and compared among testas of different permeability levels. SEM observations of the exomorphology and anatomy of the testa were made at CRIBABB (Centro Regional de Investigaciones Básicas y Aplicadas de Bahía Blanca) and CERIDE (Centro Regional de Investigación y Desarrollo de Santa Fe), using the method mentioned in [20, 21].

2.3. Observation of the cell wall of macrosclereid cells in cross sections of white clover testa

Testas were hydrated during 4 days. The fixing solution consisted of 2.5% glutaraldehyde in 0.1 M (pH 7.2) sodium phosphate buffer. A tissue section (0.5 mm long) was cut from the testa with a razor blade and immediately placed in the fixing solution for 24 h. The following steps until anatomical observations were those described in [20, 21]. Observations were made under a scanning electron microscope JEOL 100 CXII at 80 kV [22] at CRIBABB. Means (μm) were obtained from observations of nine cells of three testas per permeability level (2004 and 2006 harvests). The testas of very slow-permeability seeds that were observed and microphotographed had been immersed in water for 3000, 5000, and 7488 h and were not hydrated.

2.4. Determination of structural polymeric substances, pectic substances, and polyphenols, tannins, and cutins

The testas were selected according to their water permeability level: (a) rapid hydration (permeable). Under these conditions seeds are completely swollen and exhibit a crack in the testa due to the size increase caused by imbibed water. To obtain the testas for analysis, hydrated seeds were placed to germinate in moistened paper towel, which was rolled up, placed in nylon bags and taken to germination chamber (25°C). Rolls were placed in a 45° angle. Then, after 5 days, testas of germinated seedlings were collected, placed in Petri dishes, and left to dry in an oven (25°C) until 3 g of testas of rapid-germination seeds was obtained: (b) very low hydration (impermeable). To obtain testas of hard seeds, the seed coat was incised at the distal end of cotyledons and soaked in water until fully imbibed (4 h). Then they were placed to germinate in moistened paper towel, following the same procedure as that used for rapid-hydration (permeable) testas, until 3 g of testas of hard seeds was obtained.

Structural components of the testa cell wall were determined following the methods described in [23]: total polyphenols using the technique of Folin-Denis; condensed tannins using the vanilla method; and cell wall percentage, cell contents, and percentage of cellulose, lignin, and

cutin via the Van Soest method. Determinations were made in 0.5 g testa samples with two repetitions (approximately 3000 testas) per permeability level, using testas of both harvests [20, 21], at the Institute of Cellulose Technology, Faculty of Chemical Engineering, National University of Litoral, Santa Fe, Argentina.

3. Results and discussion

3.1. Morphological and anatomical SEM analyses of the testa

The testa of the rapid-hydration white clover seed (nondormant seeds) observed under SEM (**Figure 1**) was 30–40 μm thick, showing a thin cuticle (1μm) with slight cracks, depressions, and openings, as mentioned by [24] for the soybean testa surface. At the surface level, the ends of the macrosclereids were visible, giving an irregular and rugged appearance. Macrosclereids (**Table 1**) were approximately 30 μm long and were arranged in palisade, but not too compressed, with a visible light line. In cross section, an irregular polygonal contour was observed (three to seven sides) (**Figure 2A**). The long and short axes varied in length; cell wall width was irregular and smaller than in macrosclereids of slow-hydration testas. Osteosclereids were not visible in these seed testas, and parenchyma cells were present.

Seed coats of very slow hydration (dormant seeds) (**Figure 3**) were 45–50 μm thick and had a thick cuticle (3–5 μm), giving the surface a smooth appearance. Macrosclereids (**Table 1**) were about 40 μm long; they were arranged in a single, very compressed palisade layer and had a visible clear line. In cross section, they had an irregular, polygonal contour (three to seven sides, **Figure 2B**). The long and short axes varied in length, depending on the cell; cell wall thickness was also variable but was thicker than in permeable testa macrosclereids. Osteosclereids (**Figure 3B** and **C**) were visible, 15–18 μm in width and relatively short (5–8 μm); parenchyma cells were present. Anatomical characteristics of hard seed testas with different immersion time were similar. The anatomical characteristics of the hard seed testas of 3000 h were similar to those of 5000 h and 7488 h (data not shown). Hard seeds of this species have very deep physical dormancy [16], suggesting similar anatomical and chemical traits among seeds within that category.

3.2. Determination of structural polymeric substances, pectic substances, and polyphenols, tannins, and cutins

The results show (**Table 2**) that cellulose, hemicellulose, lignins, and condensed tannins were present in higher amounts in impermeable testas, whereas in permeable ones, a higher amount of cell content was observed. These findings are related to the presence of macrosclereids of greater size and thicker cell wall found in dormant seeds than in rapid-hydration seeds, as well as to the presence of osteosclereids.

Cellulose is associated with hemicellulose, with both being the most important structural substances in the cell wall. The cellulose values found in very low-hydration testas (48.6%)

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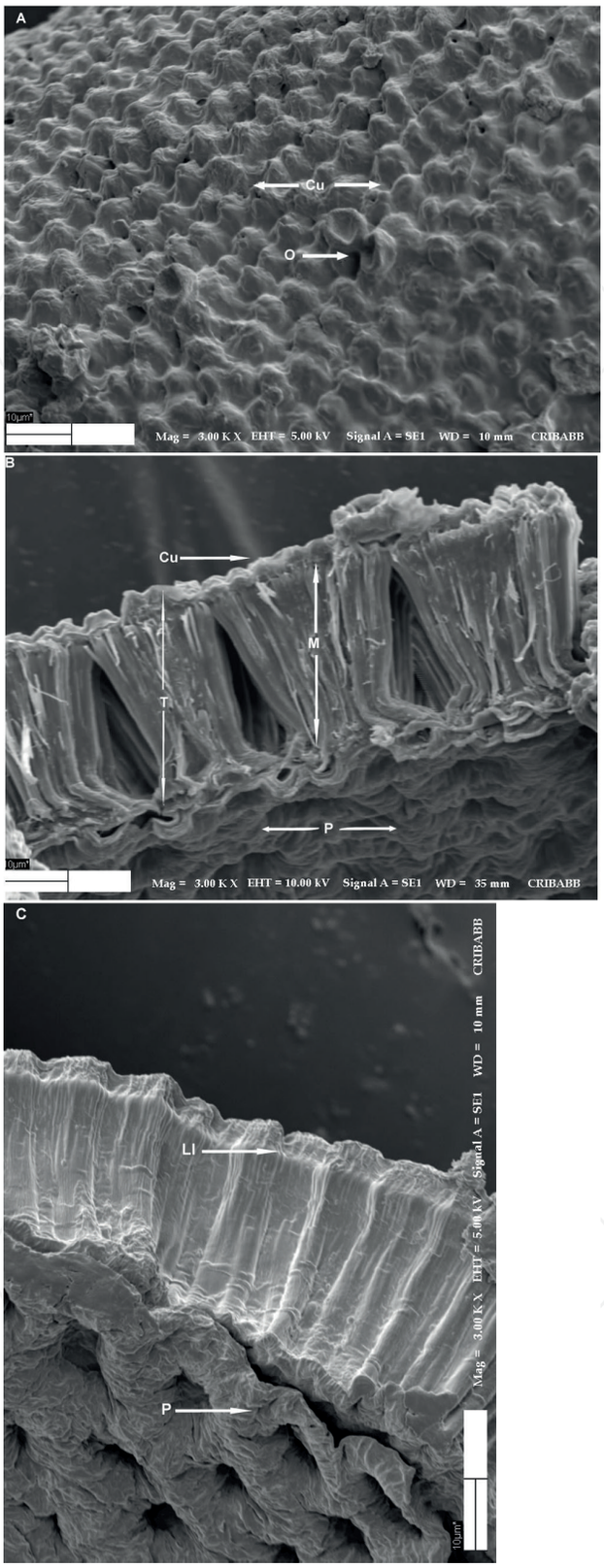

Figure 1. Testa of *T. repens* cv. NK Churrinche seed (2004 harvest) permeable after 2 h of water imbibition. (A) Testa surface. (Cu) Cuticle and (O) opening. (B and C) 3D view of testa. (T) Testa, (Cu) cuticle and waxes, (M) macrosclereids, (O) osteosclereids, (P) parenchyma, (Fs) slight fissure among cells, and (Ll) light line ×3000 (bar, 10μm).

Testa	Length (μm)	Axis (cell width) (μm)						Cell wall thickness (μm)		
		Long			Short					
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Rapid hydration	25-30	2.72	2.51	3.26	1.79	1.67	1.86	0.54	0.37	0.93
Very slow hydration	30-40	9.40	7.20	11.38	3.41	2.35	4.59	2.2	1.18	3.67

Mean values (μm) were obtained through SEM observations (×2700; ×6700 and ×10,000) of cross sections of nine cells of three teguments, 2006 harvest. (Min) minimum value; (Max) maximum value.

Table 1. Macrosclereid measurements in testa of seeds of white clover (*Trifolium repens*) cv. NK Churrinche as a function of testa permeability to water.

are close to those mentioned by [25, 26] for secondary wall (41 and 45%, respectively), with the amount of hemicellulose found (16.1%) being lower than that reported by those authors (30%). In alfalfa seeds, cellulose and hemicellulose in testas amounted to 39.9 and 20.7%, respectively [20, 21]. Although these components exhibit hydrophilic characteristics, in the cell wall of macrosclereids, lignification eventually occurs [2], forming hydrophobic secondary walls that provide rigidness [11, 27]. Lignin was found in greater amount in impermeable seed teguments than in permeable ones. This component is a highly insoluble polymer of

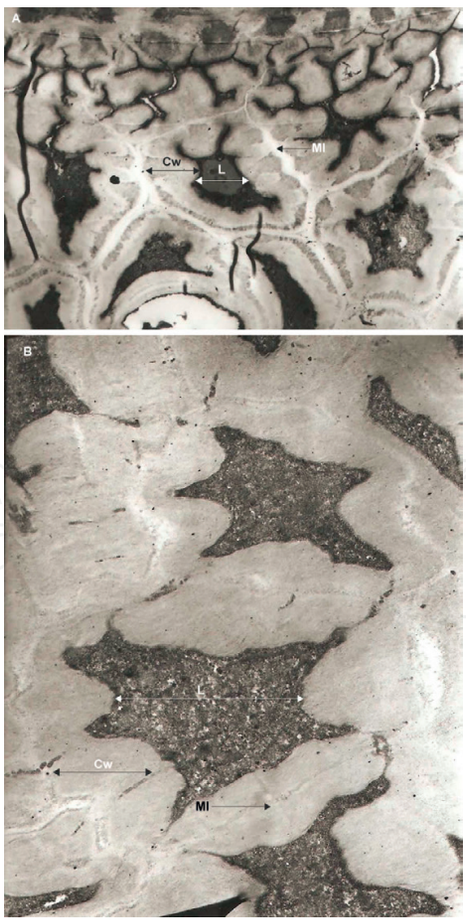

Figure 2. Macrosclereids of seed testa of *T. repens* cv. NK Churrinche (2004 and 2006 harvests) (A) rapid-hydration testa, permeable after 2 h of water imbibition. (B) Very low-hydration testa, impermeable after 3000 h water imbibition. (Cw) Cell wall, (Ml) middle lamella, and (L) cell lumen. (A) ×2700, 1 cm, 0.91μm; (B) ×6700, 1 cm, 1.31μm.

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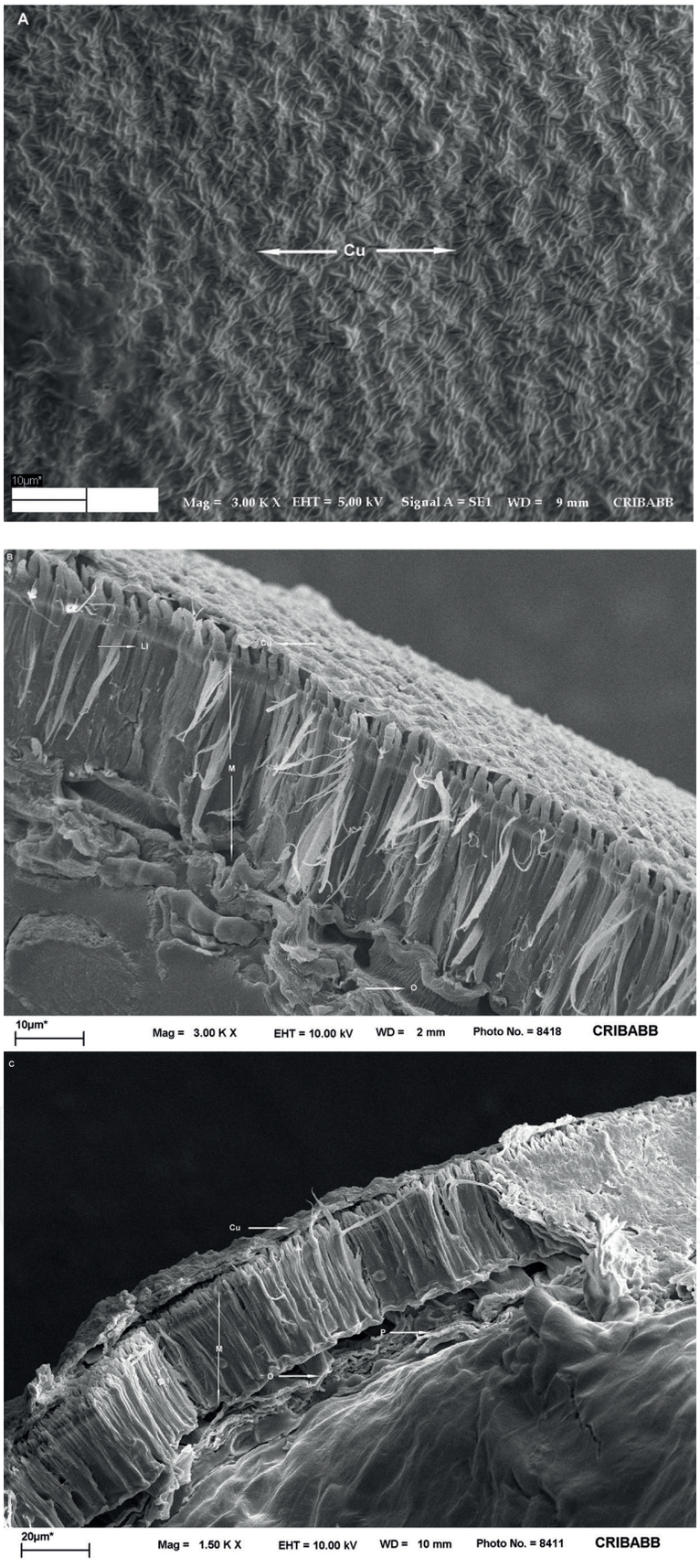

Figure 3. Testa of *T. repens* cv. NK Churrinche seed (2006 harvest) of very low hydration, impermeable after 3000 h of water imbibition. (A) Testa surface, (Cu) cuticle; (B and C) cross section of two testas, (Ll) light line, (M) macrosclereid, (O) osteosclereid, and (P) parenchyma. A and C: SEM ×3000 (A and B, bar 10 μm; C, bar 20 μm).

Testa	Tp	Ct	Cw	Cc	Cell wall		ADF		
					ADF + sílica	hc	c	l	cu
Rapid hydration	0.47	0.16	32.8	67.2	25.9	6.9	21.0	1.3	0.5
Slow hydration	0.36	0.14	69.3	30.1	56.1	13.8	45.3	3.1	9.1
Very slow hydration	–	0.29	77.1	22.9	61	16.1	48.6	5.5	*

Tp, Total polyphenols; Ct, Condensed tannins; Cw, Cell wall, (hc) hemicellulose, gums, mucilages, cellulose, lignin, and cysteine; Cc, cell content, glucose, fructose, sucrose, galactose, starch, and fructans (protoplasts and pectic substances); ADF, Acid detergent fiber. (c) Cellulose, (l) lignin, and (cu) cutin.

*It could not be determined by the extraction method; the presence was observed via SEM.

Table 2. Determination of organic compounds (%) in dry matter of seeds testa of *T. repens* cv. NK Churrinche with different permeability levels.

phenolic units that form a large network of crossed bonds. The lignin content (5.5%) found in impermeable testas is relatively high, compared to the 8% of cereals straw [28] and 16.85% of wheat straw [29]. In alfalfa, 3.34 and 7% of lignins (cell wall) were reported at leaf and preflowering stages, respectively [30, 31]. And 7.7% was integument of very slow-hydration seeds [21]. Condensed tannin is another component found in higher amount (0.29%) in hard seed testas than in permeable seeds (0.16%) and might provide the testa with astringent and feeding deterrent characteristics, as well as with defense from predators.

Regarding cell content determined in the testa (**Table 2**), a great difference was observed between rapid-hydration (67%) and very low-hydration (30.1 and 22.9%) seeds. That fraction includes diverse substances, such as pectic substances. Based on observations of the lumen in micrographs of macrosclereids, we assume that the amount of cell content determined in hard seed testas largely corresponds to pectins of the middle lamella of macrosclereids. Pectins act as cementing substances [32] and become lignified in older cells [2].

Cutin values found in rapid- and slow-hydration testas were 0.5 and 9.1%, respectively (**Table 2**). In the very slow-hydration teguments, cutin proportion could not be determined via the extraction method used; however, SEM observations showed a proportionately thicker cutin layer than in seeds of other degrees of permeability.

From an anatomical perspective, the highest level of seed physical dormancy in white clover seeds would be attributed to the combination of the effects of a thicker cuticle, thicker cell wall of macrosclereids, and greater length and width of macrosclereids than in seeds of the other permeability levels, as well as to the presence of osteosclereids.

Physiologically and chemically, the dormancy mechanism via hard coats is explained by an increased amount of hydrophobic and cementing substances, such as cutins, lignins, tannins, and pectins. Lignification of the thick cell wall would be one of the main components determining physical dormancy. White clover seeds with physical dormancy contain viable embryos and very low water content, as found in alfalfa [17]; once water permeability is produced and other appropriate factors are present, embryo imbibition occurs, originating normal seedlings [16]. The traits found in these seeds would provide physical dormancy with the capacity to preserve the embryo during the time when seeds are dormant.

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