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# Physiological quality, lignin and the ultrastructural characterization of soybean seeds

**Elisa de Melo Castro[1*], Édila Vilela de Resende Von Pinho[1], Amador Eduardo de Lima[1], Cláudia Regina Gontijo Labory[2], Heloisa Oliveira dos Santos[1], Eduardo Alves[2] and Alisson Francelino dos Reis Guerra[1]**

[1]Departamento de Agricultura, Universidade Federal de Lavras, Aquenta Sol, 37200-000, Lavras, Minas Gerais, Brazil. [2]Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil. *Author for Correspondence. E-mail: elisa.castro@ufla.br

**ABSTRACT.** In soybeans, the integument or seed coat is an important modulator between the external and internal environment. It plays a fundamental role in seed vitality, and its lignin content may influence the seed quality. The objective of this experiment was to evaluate the quality of soybean seeds from a partial diallel and their reciprocals and its relationship with lignin content, seed coat thickness, and deposition location. The seeds were also evaluated for physiological quality through germination and accelerated aging tests. The lignin content was quantified by absorbance, and the integument thickness was analyzed and measured using scanning electron microscopy. The most contrasting cultivars for lignin content were analyzed using fluorescence microscopy and histochemical techniques. Cultivars and their reciprocals differed in seed physiological quality. We found differences in the genotypes for integument thickness. Using histochemical techniques, autofluorescence was observed in the same regions identified as lignified. Positive staining for lignin was observed in the hilum region. Among the genotypes, we found variations in the physiological quality of seeds (germination and accelerated aging test), lignin content, and integument thickness.

**Keywords:** *Glycine max*; anatomy; phenolic compounds; microscopy; integument.

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## Introduction

Soybean is an agricultural crop of economic importance nationally and worldwide. Genetic improvements aiming to obtain soybean genotypes that present agronomic characteristics such as high seed quality and productivity are desirable, and they seek the best development of the field crop, especially under adverse conditions. According to Krzyzanowski and França Neto (2018), the lignin content is one of the parameters related to physiological quality, and it has been used to screen this characteristic.

There are reports of the influence of genotypes on the physiological quality of soybean seeds (Menezes, Von Pinho, José, Baldoni, & Mendes, 2009; Carvalho, Mavaieie, Oliveira, Carvalho, & Vieira, 2014; Castro, Oliveira, Lima, Santos, & Barbosa, 2016, Moreno et al., 2019).

Moreover, physiological quality has also been associated with seed coat characteristics, which are variable for different species and genotypes of the same species (Abati et al., 2022). According to Souza and Marcos Filho (2001), the seed coat is one of the main determinants of seed germination, vigor and longevity. Depending on its constitution, it can influence characteristics such vigor, storage potential, resistance to wrinkling caused by moisture damage, and infections by microorganisms.

Lignins are complex natural phenylpropanoid polymers associated with secondary plant cell walls (Chen, Tobimatsu, Havkin-Frenkel, Dixon, & Ralph, 2012). Present in the integument of seeds of several plant species, they change depending on the genetic characteristics of the cultivar, but they can also be influenced by the environment (Lewis & Yamamoto, 1990).

The use of soybean genotypes with higher lignin content in the seed coat favors the production of higher quality seeds (França Neto et al., 2016) because it affects the degree of resistance to mechanical damage (Kuchlan, Kuchlan Onkar, Ramesh, & Husain, 2018), the process of water absorption (Abati et al., 2022), and moisture damage in soybean seed cultivars (Huth et al., 2016). Many experiments have been carried out to understand the influence of lignin in the seed coat of soybean seeds and the physiological characteristics associated with lignin content (Castro et al., 2019; Botelho et al., 2019; Baldoni, Von Pinho, Fernandes, Abreu,

& Carvalho, 2013; Carvalho, Oliveira, & Caldeira, 2014). Ultrastructural and histochemical analysis of these integuments can elucidate aspects related to lignin and the physiological quality of soybean seeds

The analysis of these botanical structures can be done using electron microscopy, which is an important method in ultrastructural studies. It allows us to understand the internal relationships in plant tissues with other important characteristics. However, studies involving electron microscopy combined with histochemical techniques in different genetic materials of soybean seeds where there are variations in lignin content are scarce in the literature. Thus, the objective of this research was to evaluate the quality of soybean seeds from a partial diallel and its reciprocals and its relationship with the integument thickness, location, and lignin contents in the integument.

## Material and methods

### Location

The experiments were conducted at the Technology Development and Transfer Center, municipality of Ijaci, in Minas Gerais State, in the Central Seed Laboratory of the Department of Agriculture and Multiuser Laboratory of Electronic Microscopy and Ultrastructural Analysis of the Federal University of Lavras (UFLA), in Lavras, Minas Gerais State, Brazil. The weather field conditions were at the latitude 21°09'51.6" S, longitude 44°55'00" W, and altitude of 833 m during the growing season.

### Genotypes and field conditions

Six soybean cultivars were evaluated and divided into two groups, first classified according to Moreno et al. (2019) by germination and vigor tests (accelerated aging, first germination count, final count germination, and controlled deterioration) in a study carried out previously. Group one (G1) had three cultivars with seeds of high physiological quality (CD 201, CA 115, and MS 8400) and group two (G2) had three cultivars with seeds classified as low physiological quality (CD 202, Syn 1263, and Syn 1279). The seed sowings were carried out at six different times to ensure the coincidence of flowering among parents. These two groups were manually intercrossed, using the partial diallel crossing system.

The field experiment was installed in an area under semi-protected cultivation using plastic film to cover the ceiling part of the structure, but both sides were uncovered. Each plot consisted of five lines of five meters in length and spaced 0.5 m apart. The usable area of the plot was 3 $m^2$, considering the central region of the three central rows. The sowed seeds were treated at the time of the test with the fungicide Vitavax-Thiran 200 SC, applying the dosage of 250 mL 100 $kg^{-1}$ of seeds. Sowing fertilization, soil analysis, and interpretation in accordance with the recommendations for soybeans, in Ribeiro, Guimarães and Alvarez (1999), were considered. The water source was supplied by dripping irrigation. The high temperature was 34.5°C and the low temperature during the experimental time was 19°C. Seeds from crosses and their reciprocals were harvested at stage R8 and dried under shade until the water content was approximately 12%. To evaluate the characteristics, we used the $F_2$ generation which was obtained by self-pollinating the $F_1$. After manual threshing, the seeds were classified by sieves of 5.55 and 6.35 mm and then selected for further evaluation.

### Laboratory conditions

### Seed quality analysis

Seed quality was determined using germination and vigor tests. For each of them, four replications of 50 seeds from 24 treatments were evaluated with six lines and 18 populations from each cross and its reciprocal.

For the germination test, the seeds were sown in germitest paper (three sheets) moistened with distilled water 2.5 times the weight of the dry paper. Then they were germinated at 25°C (Brasil, 2009). The evaluations were carried out at five days (first count) and eight days after the installation of the test (final count), and the results were expressed as the percentage of normal seedlings (Brasil, 2009).

The accelerated aging tests were conducted in gerbox transparent plastic boxes. The seeds were placed in a single layer on the wire screen suspended under every box containing 40 mL of distilled water. These boxes remained at 42°C for 82 hours in the BOD incubator; this period was determined based on preliminary tests. Next, the seeds were submitted to the germination test described above.

## Lignin content analysis

The evaluation of the lignin content in the seed coat was carried out in the 24 genotypes using four replicates of 50 seeds. The integuments were removed and macerated in a crucible using liquid nitrogen, and the lignin extraction was performed according to the methodology described by Capeleti et al. (2005). To determine the lignin content, dry residues insoluble in methanol containing lignin and esterified phenolic acids from the cell wall were used. The lignin solution obtained at the end of the extraction was evaluated by absorbance at 280 nm, the values were estimated based on the lignin curve and expressed in mg of lignin per gram of dry tissue.

## Scanning electron microscopy (SEM)

Integument samples of ten seeds from each of the 24 genetic materials were collected and removed with tweezers and a scalpel. The seeds were sectioned transversal to the hilum, standardizing the region (Figure 1A). Next, they were set in an aluminum stub and covered with gold using the Balzers SCD 050 sputtering device (Balzers, Liechtenstein) for observation under a scanning electron microscope LEO EVO 40 (SEM) (Carl Zeiss, Oberkochen, Germany). The region of observation was standard, and measurements were performed between the hilum and the region opposite to the hilum (Figure 1B). The images were used to measure the total thickness and the individual layers of the integument, with 14 measurements per layer, per genotype.

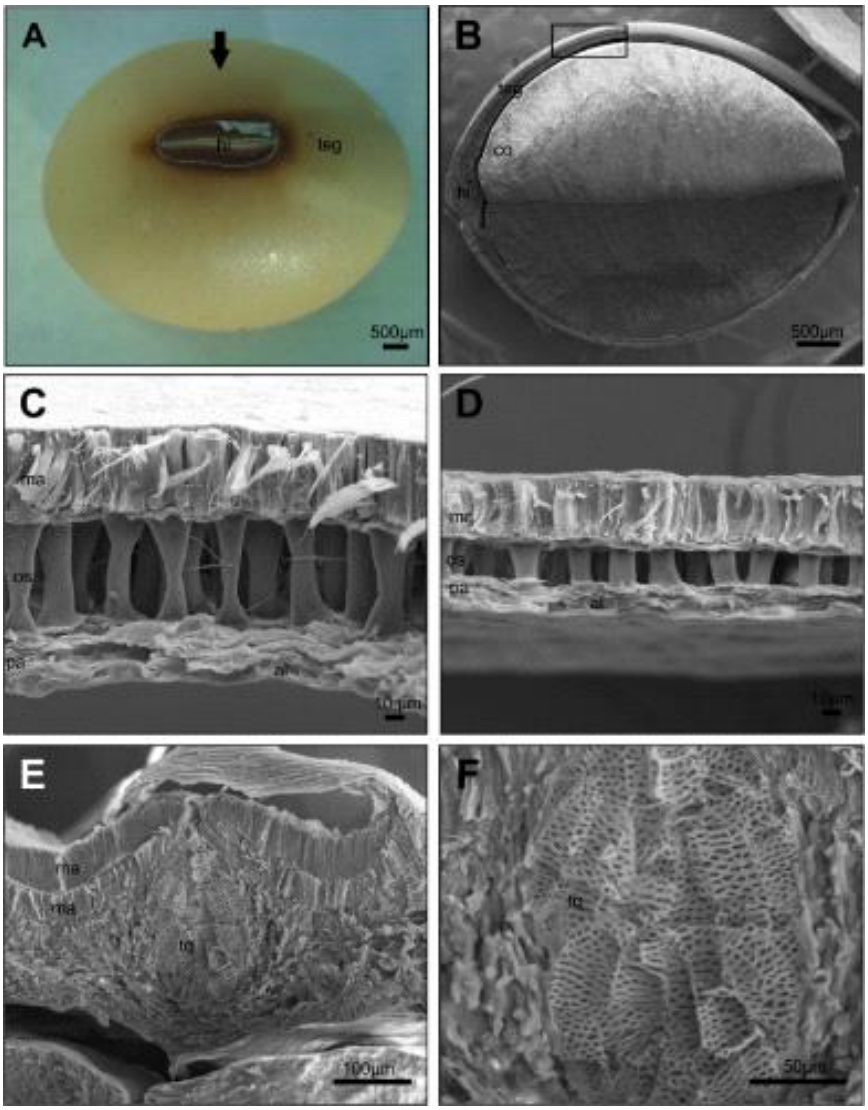


**Figure 1.** Soybean seed photomicrograph: A: indication of the section location for sample assembly; scanning electron micrograph of soybean seed integuments (B-F). B: Sectioned soybean seed evidencing the analyzed area standardization for integument thickness. C: Soybean integument from the higher thickness crossing (MS 8400(F) x CD 202(M)). D: Soybean integument from the lower thickness crossing (CD 202(F) x CD 201(M)). E: Hilum structure in transversal cut evidencing tracheoids and two macrosclereids layers. F: detail for reticulated ornamentation for tracheoids wall. hi: hilum, teg: integument, ma: macrosclereids, os: osteosclereids, pa: parenchyma, al: aleurone, tq: tracheoids.

### Histochemical analysis

The seeds of the cultivars CD 201 and Syn 1263 were selected in order to locate the lignin in the seed coat having higher and lower lignin content, respectively, and because they have more contrasting characteristics, as quantified by the method of Capeleti et al. (2005).

The seed coats were carefully removed by tweezers and a scalpel, placed on a metallic support using gum Arabic glue, and frozen at -18ºC. Subsequently, they were sectioned at 20 µm using a Leica Ag Protect CM 1860 Cryostat.

The integument sections were set up on glass slides and observed under a light microscopy (no staining) using histochemical marking. The hilum region was analyzed as well as the same region described for the SEM analysis (Figure 1A and B).

For histochemical characterization, the sections were set up on glass slides, stained with phloroglucinol (2%) (phloroglucionol, ethanol 95%), then placed in hydrochloric acid 25% (hydrochloric acid and distilled water) and immediately observed in a light microscope. Lignin deposition was observed with a pink to intense red coloration (Johansen, 1940; Ventrela, Almeida, Nery, & Coelho, 2013). Sections set up on glass slides were also stained with toluidine blue (0.05%), and the structural phenolic compounds (lignins) were identified by blue staining tending to green (O'brien, Feder, & McCully, 1964; Ventrella et al., 2013). The Carl Zeiss inverted observer Z1 Epi-Fluorescence Microscope was used.

### Autofluorescence analysis

Slices taken from the seed coats of the cultivars CD 201 with higher and Syn 1263 with lower lignin content were obtained in the same way as the histochemical analysis. We observed autofluorescence using a blue (excitation 426-446 nm, emission 460-500 nm), green (excitation 450-490 nm, emission 500-550 nm), and red (excitation 530-560 nm, emission 590-650 nm) excitation cube from the Carl Zeiss inverted observer Z1 Epi-Fluorescence Microscope.

### Statistical analysis

All data were submitted to the Shapiro and Wilk (1965) normality test to verify the need of data transformation. Data from the first and final germination counts did not show normality and were transformed to arcsine for further analysis. The averages of the germination, accelerated aging test, and lignin tests were submitted to the Scott-Knott test at 5% of probability using the Genes software (Cruz, 2013). The lignin thickness tests were submitted to the Scott-Knott test at 5% probability, using the Sisvar software (Ferreira, 2011). The results of the histochemical analyzes were qualitatively evaluated through image observation.

Pearson's correlations were used to evaluate the interaction of the accelerated aging test (EA), lignin (L), first germination count (G1), germination (G) and thicknesses of the palisade (Pal), hourglass (HG), aleurone (Al), parenchyma (Par), and total tegument layers of the studied soybean seeds. Levels of $p < 0.05$ and $p < 0.01$ were considered significant and highly significant, respectively. Pearson's correlation coefficient was interpreted and classified according to Dancey and Reidy (1999). Data processing and analysis were performed in the R environment (R Core Team, 2020) and RStudio (1.4.1106 version).

## Results and discussion

We found significant differences in the analyzed variables (Table 1). In seeds with higher lignin content, higher physiological quality was not necessarily found. In seeds from the cultivar CD 201, higher lignin content was found when compared to the other cultivars. However, for most of the crosses that had this cultivar as a female or male parent, this characteristic seems not to have influenced the physiological quality of seeds evaluated by germination and accelerated aging tests.

In cultivars initially classified by seeds with high (CD 201, CA 115, and MS 84000) or low (CD 202, Syn 1263, and Syn 1279) physiological quality, higher or lower quality, respectively, was not found in this research. Possibly, this is because of the influence of environmental factors and interaction between factors since physiological quality is a characteristic controlled by many genes and greatly influenced by the environment.

Seeds of the genotypes CA 115 and CD 202, with intermediate levels of lignin, and those of the crosses, CD 202(F) x MS 8400(M), CD 202(F) x CD 201(M), CA 115(F) x CD 202(M), Syn 1279 (F) x CD 201(M) with lignin contents ranging from intermediate to high among the studied genotypes (0.325 to 0.600 mg $g^{-1}$), were found to perform better for initial and final germination and vigor, as evaluated by the accelerated aging test. Seeds

of the two cultivars with the highest quality are present in most of the crosses with the highest quality, which was not observed from the seeds of the reciprocals. Thus, these results should suggest to breeders some advantages of performing crosses, giving priority to female genitors with better physiological quality.

**Table 1.** Averages from the accelerated aging tests (%) (AA), first germination count (G1) and germination (G), lignin content (mg $g^{-1}$) (L), thickness of the cell layers of the tegument (µm): palisade cells (Pal.), hourglass cells (HG), parenchyma (Par.), aleurone (Aleu.), and total.

| Genotypes | Physiological quality | | | | Integument thickness | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AA | G1[1] | G[1] | L | Pal. | HG | Par. | Aleu. | Total |
| | % | | | mg $g^{-1}$ | µm | | | | |
| CD 201 (G1) | 41 f | 92 b | 97 b | 0.587 b | 44.902 a | 33.346 b | 12.500 a | 7.197 d | 97.947 b |
| CA 115 (G1) | 99 a | 98 a | 100 a | 0.410 c | 37.693 c | 31.303 b | 11.830 a | 6.776 d | 87.602 c |
| MS 8400 (G1) | 46 e | 49 e | 61 d | 0.370 d | 40.901 b | 39.694 a | 11.152 a | 7.259 d | 99.007 b |
| CD 202 (G2) | 99 a | 98 a | 100 a | 0.405 c | 35.273 d | 40.572 a | 10.841 a | 8.658 c | 95.346 b |
| Syn 1263 (G2) | 88 b | 98 a | 99 a | 0.215 h | 41.537 b | 46.696 a | 12.370 a | 10.704 a | 111.308 a |
| Syn 1279 (G2) | 78 c | 92 b | 97 b | 0.310 f | 38.325 c | 42.023 a | 12.364 a | 10.578 a | 103.292 a |
| Syn 1263 (F) x CA 115(M) | 29 g | 92 b | 97 b | 0.202 h | 38.487 c | 40.476 a | 11.659 a | 11.110 a | 101.733 a |
| CA 115 (F) x Syn 1263(M) | 79 c | 94 b | 99 a | 0.400 c | 40.066 b | 43.527 a | 12.988 a | 11.513 a | 108.096 a |
| Syn 1263(F) x MS 8400(M) | 51 e | 91 b | 98 a | 0.217 h | 38.836 c | 35.646 b | 12.614 a | 9.561 b | 96.658 b |
| MS 8400(F) x Syn 1263(M) | 40 f | 70 d | 85 c | 0.355 d | 41.610 b | 42.195 a | 11.472 a | 10.719 a | 105.997a |
| Syn 1263(F) x CD 201(M) | 52 e | 85 c | 97 b | 0.225 h | 41.584 b | 44.461 a | 14.054 a | 9.036 c | 109.136 a |
| CD 201(F) x Syn 1263(M) | 23 g | 65 d | 77 c | 0.615 a | 39.517 c | 41.273 a | 11.644 a | 9.012 c | 101.448 a |
| CD 202(F) x MS 8400(M) | 98 a | 97 a | 100 a | 0.420 c | 33.928 d | 34.598 b | 12.012 a | 8.771 c | 89.310 c |
| MS 8400(F) x CD 202(M) | 58 d | 85 c | 94 b | 0.363 d | 45.306 a | 48.275 a | 15.331 a | 10.683 a | 119.597 a |
| CD 202(F) x CD 201(M) | 97 a | 99 a | 100 a | 0.600 b | 36.250 c | 30.917b | 8.5470 a | 8.839 c | 85.666 c |
| CD 201(F) x CD 202(M) | 33 f | 67 d | 85 c | 0.378 d | 42.830 a | 43.971 a | 11.399 a | 9.726 b | 107.927 a |
| CD 202(F) x CA 115(M) | 88 b | 96 b | 100 a | 0.448 c | 34699 d | 34.918 b | 9.7757 a | 9.943 b | 89.337 c |
| CA 115(F) x CD 202(M) | 97 a | 98 a | 99 a | 0.408 c | 36250 d | 39.535 a | 12.857 a | 9.356 b | 98.000 b |
| Syn 1279 (F) x CA 115(M) | 59 d | 87 c | 95 b | 0.260 g | 40174 b | 41.466 a | 11.902 a | 10.927 a | 104.471 a |
| CA 115(F) x Syn 1279(M) | 65 d | 93 b | 98 b | 0.428 c | 34328 d | 36.883 b | 11.853 a | 9.755 b | 92.821 b |
| Syn 1279(F) x MS 8400(M) | 81 c | 96 a | 97 b | 0.303 f | 38.420 c | 47.621 a | 11.233 a | 11.219 a | 108.494 a |
| MS 8400(F) x Syn 1279(M) | 53 e | 84 c | 89 c | 0.373 d | 40.234 b | 37.944 b | 11.717 a | 9.627 b | 99.523 b |
| Syn 1279(F) x CD 201(M) | 91 a | 93 b | 99 a | 0.325 e | 40.497 b | 45.459 a | 12.488 a | 11.241 a | 109.686 a |
| CD 201(F) x Syn 1279(M) | 36 f | 83 c | 93 b | 0.638 a | 42.100 b | 38.610 b | 10.916 a | 9.844 b | 101.472a |
| CV (%) | 10.655 | 6.85 | 3.9 | 4.8 | 12.55 | 22.47 | 20.73 | 30.03 | 14.34 |

*Averages followed by the same letters, in the columns, do not differ by the Scott-Knott test at 5% probability.

Considering only the initial and final germination tests, seeds of the cultivar Syn 1263 and of the crosses Syn 1263(F) x MS 8400(M) and Syn 1279(F) x CD 201(M), with lignin contents less than 0.325 mg $g^{-1}$, higher than 91% germination was observed. In this case, the decrease in lignin did not necessarily result in a decrease in germination.

In the AA test, there was low vigor of several genotypes, including some with high germination, such as CD 201 and Syn 1263(F) x CA 115(M). The AA test has been used as a genotype differentiator. Similarly, Saini et al. (2022) studied the genetic control of soybean seed viability and its association with other traits through AA. There is a strong positive correlation between accelerated aging and first-count germination and germination (Table 2).

**Table 2.** Pearson's correlations of the accelerated aging test (AA), lignin (L), first germination count (G1), germination (G) and thicknesses of the palisade (Pal), hourglass (HG), aleurone (Aleu), parenchyma (Par) and total tegument layers of the studied soybean seeds.

| | AA | L | G1 | G | Pal | HG | Aleu | Par | Total |
|---|---|---|---|---|---|---|---|---|---|
| AA | 1 | | | | | | | | |
| L | -0.06 | 1 | | | | | | | |
| G1 | 0.69** | -0.03 | 1 | | | | | | |
| G | 0.66** | -0.09 | 0.88** | 1 | | | | | |
| Pal | -0.23* | 0.05 | -0.28** | -0.23* | 1 | | | | |
| HG | 0.10 | -0.15 | 0.09 | 0.05 | 0.17 | 1 | | | |
| Aleu | 0.02 | -0.28** | 0.05 | 0.11 | 0.17 | 0.30** | 1 | | |
| Par | 0.03 | -0.16 | 0.01 | 0.02 | 0.34** | 0.27** | -0.06 | 1 | |
| Total | -0.03 | -0.11 | -0.03 | 0.00 | 0.36** | 0.43** | 0.26* | 0.31** | 1 |

*Significant at $p < 0.05$; ** significant at $p < 0.01$.

According to some authors, lignin may be associated with susceptibility to mechanical damage (Capeleti, Ferrarese, Krzyzanowski, & Ferrarese-Filho, 2005; Kuchlan et al., 2018), potential deterioration (Baldoni et al., 2013), water absorption capacity, water absorption speed, and impermeability, among others (Gris, Von Pinho, Carvalho, Diniz, & Andrade, 2016; Abati et al., 2022). Also, it may be correlated with electrical conductivity (Panobianco, Vieira, Krzyzanowski, & França Neto, 1999).

Regarding moisture damage, Huth et al. (2016) found that cultivars with higher lignin content in the seed coat were less susceptible to this type of damage. These results corroborate those observed by de Castro et al. (2016), when they simulated pre-harvest rain conditions. In this work, field crop management conditions were carried out in semi-protected irrigated cultivation, which may have influenced the response of seeds to lignin content, in the sense that they were not exposed to adverse weather conditions. Bellaloui (2012) verified that environmental conditions of water stress (plants were kept between −90 and −100 kPa) promoted an increase in the lignin content in the seed coat of soybeans.

Bellaloui, Smith, and Mengistu (2017) reported that the lignin present in the seed coat is one of the main components that influence the seed quality, and it is related to germination, hard seeds, water permeability, and resistance to deterioration. However, Botelho et al. (2019) observed a negative relationship between the lignin content in the integument and the physiological quality of soybean seeds. Higher physiological quality was observed in seeds of cultivars with lower lignin content in the integument. In this research, no correlation was found between lignin content and the germination and vigor tests (Table 2). Thus, despite the lignin present in the integument having been investigated, the results found in the literature do not converge into a common understanding, suggesting that the control of this characteristic is not yet completely understood and elucidated.

Seeds from cultivar crossings and their reciprocals were different regarding the integument total thickness and also for palisade cells, hourglass, and aleurone (Table 1). For crossing MS 8400(F) x CD 202(M) seeds, the thickness was high (119.59 µM), and it was lower for crossing CD 202(F) x CD 201(M) (85.66 µM) (Table 1 and Figure 1C and D). The average integument thickness among the 24 studied genotypes was 100.49 µM.

For seeds from the crossing Syn 1279(F) x CD 201(M), higher thickness was observed for the integument and also better physiological quality and intermediary lignin content. However, better quality was not observed in most of the genetic material with higher integument thickness, and higher physiological quality was observed (Table 1) for seeds from the crossing CD 202(F) x CD 201(M), with lower thickness, but no correlation was observed between total tegument thickness and seed quality (Table 2).

These results are similar to those reported by Menezes et al. (2009) for the effects of a genetic additive and not an additive for integument layers thickness and soybean seed lignin content. The authors could not establish a correlation between seed physiological quality and integument anatomic aspects, evidence that strengthens the complexity of soybean physiological quality characteristics, which is influenced by many factors. When Giurizatto, Souza, Robaina, and Gonçalves (2003) analyzed soybean genotypes observed that integument thickness alone was not a characteristic that assured seed physiological quality.

Thickness may be correlated with integument genetic characteristics. Mertz et al. (2009) detected differences between integument structures for soybeans with black and yellow color. Those with black integument had higher thicknesses, especially in the epidermis and hypodermis.

Kuchlan, Dadlani, and Samuel (2010), studied soybean integument using scanning electron microscopy, among other techniques. They observed that genotypes with thicker integument, with less space between cotyledons and tegument, have more lignin content and better hourglass cell distribution. This provides more mechanical resistance, which may be one of the main factors that determine soybean seed longevity. According to Brzezinsk et al. (2022), the thickness of the hypodermis of the testa is related to resistance to weathering deterioration and to the obtainment of high-quality seeds.

Soybean integument cell layers were visualized in detail through scanning electron microscopy (Figure 1C and D). Palisade cells were observed (epidermis or macrosclereids), which are elongated, perpendicular to the seed surface, and have thick walls. The hypodermis (hourglass cells or osteosclereids) was also observed. It presents a single layer separated by large intercellular spaces and parenchyma cells, formed by tangentially elongated cells uniformly distributed. The aleurone layer was also observed. These results corroborate with those described by Souza and Marcos Filho (2001), Moïse et al. (2005), Miller, Bowman, Gijzen, and Miki (1999), Miller et al. (2010), and Brzezinski et al. (2022). According to Gloria and Carmello-Guerreiro (2006), a seed can be denominated testal when the main mechanical tissue layer, which is composed of thick wall cells but not necessarily lignified, is present in the external epidermis (integument). This classification may be

subdivided into exotestal, mesotestal, and endotestal. Exotestal happens when the external epidermis forms a rigid palisade layer (exotestal) composed of macrosclereids cells that is thick, lignified, or not. Based on this anatomic information (Figures 1, 2, and 3), it was verified that soybean seeds can be considered as part of an exotestal group.

For palisade cells, four distinguishable groups of average statistical differences were observed. For cultivar CD 201 seeds and crossings MS 8400(F) x CD 202(M) and CD 201(F) x CD 202(M), higher thicknesses were observed. A relationship between this layer thickness and its parent was not observed. Seeds from the crossings of cultivar CD 201 were not observed with higher thickness values and for those from cultivar CD202, which were grouped among the genotypes with lower palisade cell thickness. There were two crossings with higher palisade cell thickness (Table 1). For these genetic materials with higher palisade cell thickness, intermediary lignin content was observed (0.363 a 0.587 mg $g^{-1}$). For leguminous plants, the chemical composition, arrangement, and the palisade layer intercellular substances can influence the seed water absorption (Carvalho & Nakagawa, 2012; Baskin & Baskin, 2014).

For osteosclereids cells, averages were sorted into two distinct groups. For cultivars MS 84000, CD 202, Syn 1263, Syn 1279, and eleven other crossings, higher thicknesses were observed for hourglass cells, varying from 39 to 48 μm, and, for the remaining genotypes, there were lower averages for this cell layer, which varied from 30.9 to 38.6 μm (Table 1). Offspring with higher thicknesses were observed in seeds from many cultivars sorted into the lower thickness category for this layer. Osteosclereids or hourglass cells present in the seed coating can act as a protein storage (Moïse et al., 2005) location. Hourglass cell thickness correlates positively with layer thickness in aleurone, parenchyma, and total (Table 2).

Also denominated as columns or pillars, osteosclereid cells are formed by a subepidermal layer with visible spaces filled with air, differentiated in osteosclereids (bone-forming cells) (Smýkal, Vernoud, Blair, Soukup & Thompson, 2014).

According to Ferreira, Franke, and Moco (2011), macrosclereid cells (palisade) and osteosclereids (hourglass) present cell content rich in phenolic compounds such as lignin. However, for this research, no positive correlation between lignin content and hourglass cell thickness was found (Table 2).

The presence of tangential elongated parenchymatic cells was found at the innermost part of the soybean seed coatings (Figure 1C and D), and there was no variation in the thickness for the studied genotypes (Table 1). During the seed maturation process, these cell tissues lose protoplast, and the innermost layers may be crushed, and, generally, they are not associated with water impermeability (Smýkal et al., 2014). This parenchymatic region is also known as the "nutrient layer" due to its function during embryo development (Van Dongen, Ammerlaan, Wouterlood, Van Aelst, & Borstlap, 2003).

There was a distinction for aleurone layer thickness, but direct and conclusive relationships between this thickness and physiological quality were not observed, while for lignin, the correlation was significant and negative (Tables 1 and 2).

During the process of soybean seed coat development, as the seed coat matures, the cell layers of the endosperm adjacent to the embryo degenerate, and the outermost layer of the endosperm remains intact, differentiating into what is known as the aleurone, the only clearly visible part of the endosperm (Miller et al., 1999). Ma, Cholewa, Mohamed, Peterson, and Gijzen (2004) observed that aleurone cells have dense protoplasts and were the only alive cells in seed coating.

In the chalazar region, hilum can be observed. It is formed by a double palisade macrosclereids layer, a tracheoid layer, and a parenchyma (Figures 1E, 1F, 2A, and 2D). The hilum is an abscission scar, generally in oval or round form, resulting from the seed-maternal plant link through the funicle (Smýkal et al., 2014). It was evidenced that tracheoids presented a vertical arrangement (Figures 1F, 2A and D). This standard is commonly found in fabaceous (Lersten, 1982), and it was observed in *Adesmia tristes* and other Fabaceae species (Ferreira et al., 2011).

In Figure 2, the presence of two macrosclereids cell layers, which are not observed in the extra-hilar region, can be observed in soybean tegument sections close to the hilum.

For the integument of the two cultivars, with higher (CD 201) and lower (Syn 1263) lignin content, a greenish color was observed in the hilum region when treated with toluidine blue (Figure 2B and E). The color was more intense and characteristic at the tegument from the cultivar with more lignin content, mainly at the hilum region, strongly marked in the tracheoids and macrosclereids innermost layer. In the same region, there was a pinkish/reddish color when phloroglucinol was used (Figure 2C and F), evidencing that both techniques were efficient and complementary for identifying the lignified region.

The lignin deposition into the integument is more concentrated in the hilum region (Figure 2B, C, E, and F) when compared to the extra-hilum region (Figure 2H, I, K, and L). Ma et al. (2004) also observed this lignin deposition around the hilar fissure, tracheids, and palisade layer. The hilum region is involved in embryo water absorption (Geisler, Pinto, Santos, & Paulilo, 2017). These results can help in study comprehension, as Botelho et al. (2019) verified that for cultivars with less lignin content, the germination percentage is a little higher than that observed for those with higher content.

Chachalis and Smith (2001), studying soybean imbibition, also realized a histochemical test using phloroglucinol in HCl and, as in the present study (Figure 2H, I, K, and L), none of the seed abaxial coating regions had the typical lignin color.

For most of the Fabaceae species, according to Geisler et al. (2017), coating water impermeability is the result of one or more elongated and lignified Malpighian cell layers, which are compacted and impregnated with water repellent components.

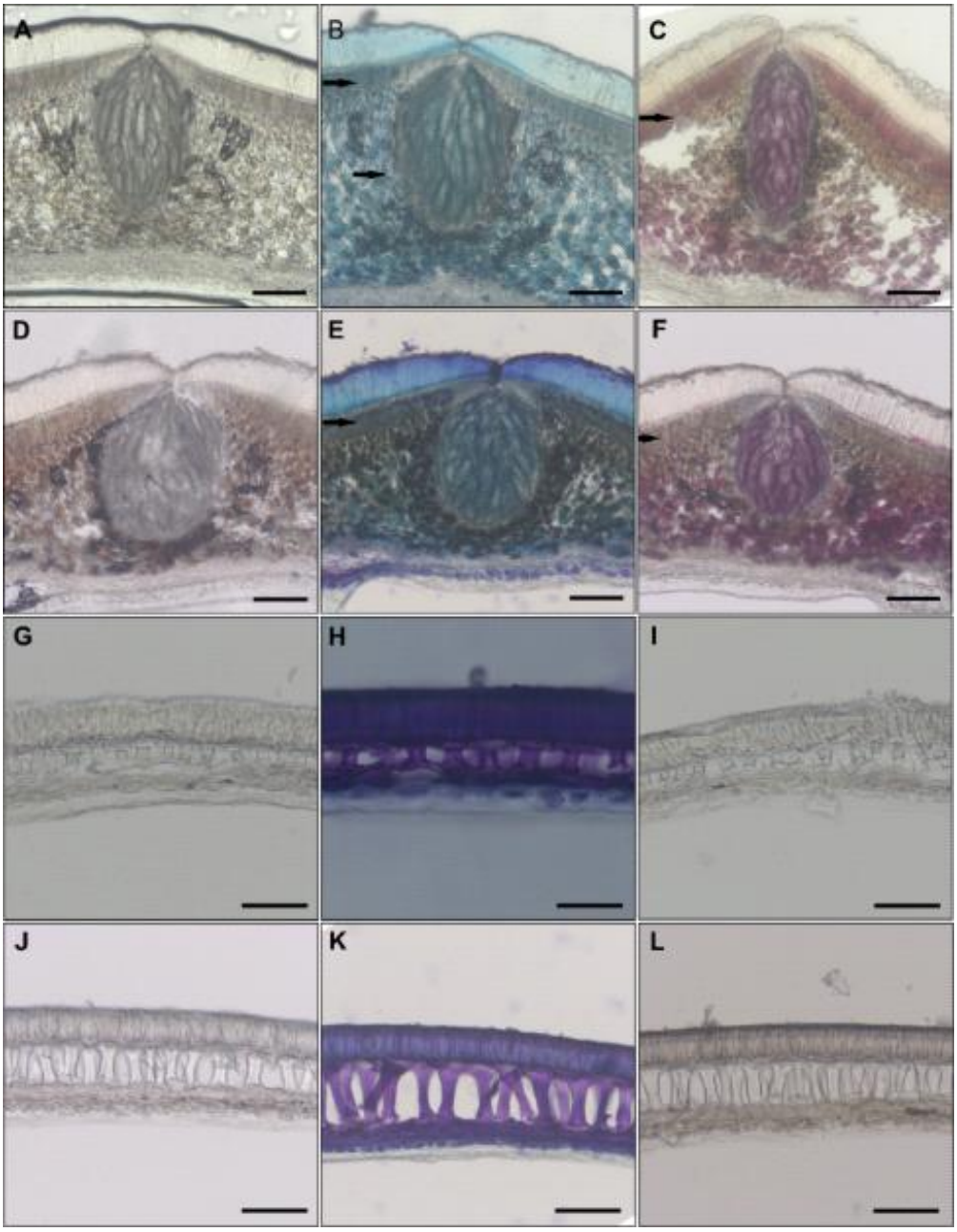


**Figure 2.** Photomicrographs of transversal sections of soybean integument. A, B, and C: Cultivar CD 201 seed hilum, with more lignin content, colored with toluidine blue and phloroglucinol, respectively. D, E, and F: Cultivar Syn 1263 hilum, with less lignin content, colored with toluidine blue and phloroglucinol respectively. G, H, and I: seed coat of soybean cultivar CD 201, with higher lignin content, stained with toluidine blue and phloroglucinol, respectively. J, K. and L: Cultivar Syn 1263, with less lignin content, colored with toluidine blue and phloroglucinol, respectively. Scale bars = 100 μm.

Integuments from the cultivars with the lower lignin content have autofluorescence when submitted to excitation in the wavelengths of blue, green, and red filters (Figure 3). However, the signal intensity was subtle in the blue exposition for both genotypes (Figure 3A and D). It was observed that the most evident fluorescence was observed in the cultivar CD 201 integument, with more lignin content, in both green and red exposition. Signal intensity was higher in the macrosclereids layer and tracheoid (Figure 3B, C, E, and F).

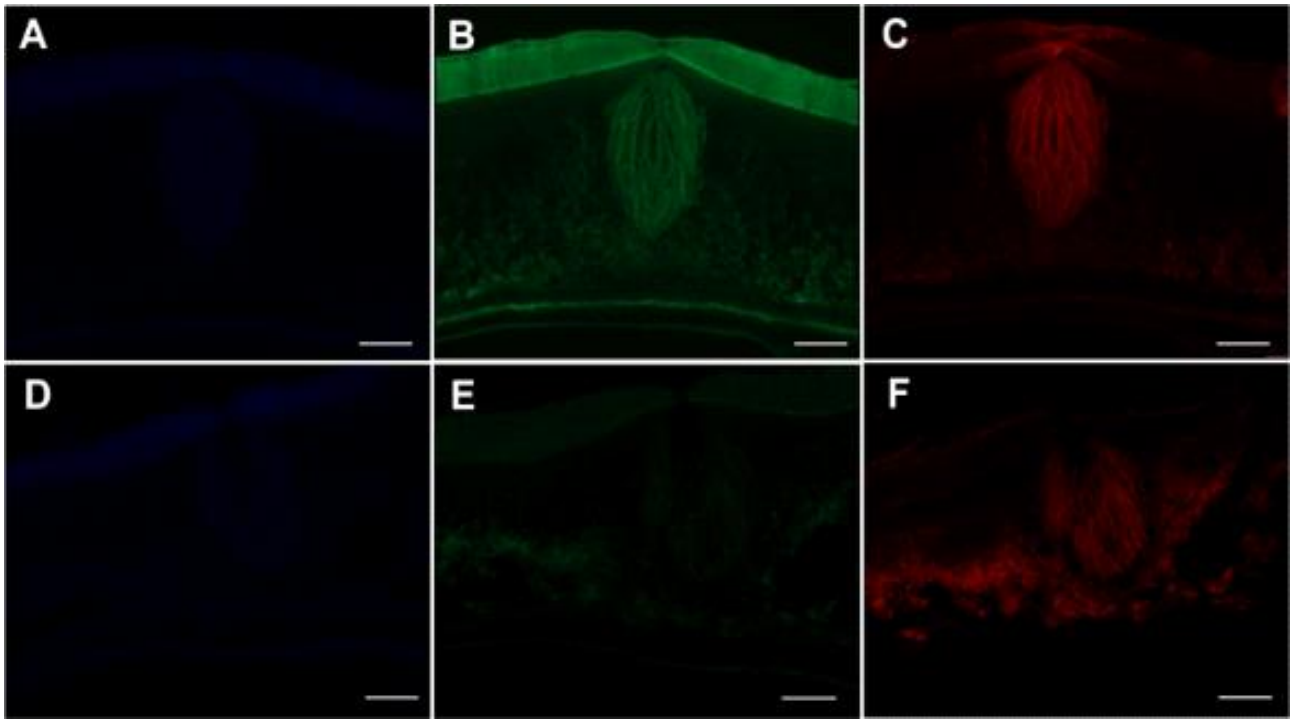


**Figure 3.** Photomicrograph of the epifluorescence intensity from soybean seeds using the blue, green, and red filters. Seeds from the cultivar CD 201 with more lignin content A, B, and C. Seeds of the cultivar Syn 1263 with less lignin content D, E, and F. Scale bars: 100 μm.

Through the autofluorescence results and histochemical analysis (Figures 2 and 3), was possible to observe higher lignin concentrations in the hilum lignified regions.

Lignin present at plant cell walls can exhibit autofluorescence under UV or visible excitation (Donaldson & Radotic, 2013). Sampaio, Abreu, Augusto, Silva, and Ibanez (2016) observed lignin autofluorescence in araucaria seed integument, allowing identification of macrosclereids highly lignified at the exotestal by using a green excitation filter (330-385 nm).

Seed quality is influenced by a set of genotype inherent factors. In this research, it was observed that, among the studied genotypes, there was a variation in this characteristic and that they also differed regarding the tegument layers thickness. In the literature, research comparing seed physiological quality, lignin content, and ultrastructural characterization is scarce. However, among the results observed for this research, histochemical and green wavelength autofluorescence techniques are highlighted. They allow us to verify higher lignin concentrations at the hilum region. Thus, additional studies aiming to elucidate the factors which interfere with seed physiological quality are important.

## Conclusion

We found different physiological quality and integument structures in soybean seeds from crossing cultivars with contrasting quality and their reciprocals. Seeds with higher lignin content did not necessarily have higher physiological quality and integument thickness. The lignin deposition was concentrated more at the hilum than at other integument regions.

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