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ARTICLE

Lignin monomeric composition in soybean seed coats and resistance to mechanical damage

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ABSTRACT: Soybean seeds are crucial for global food production. Various factors affect the quality of soybean seeds, including mechanical damage, which can lead to reduced germination potential and decreased seedling vigor. The presence of lignin in the seed coat contributes to resistance to mechanical damage. However, the relationship between the monomeric composition of lignin and mechanical damage is unknown. To fill this gap, we evaluated the contents of monomers hydroxyphenyl (H), guaiacyl (G), and syringyl (S) in seed coats of three cultivars of soybean, namely, Doko, IAS-5, and Savana. The results revealed that the monomeric composition of lignin varied between resistant and susceptible cultivars. The levels of G and S monomers were inverse in the cultivars Doko and Savana, suggesting that the composition of lignin monomers may play a crucial role in the resistance of soybean seeds to mechanical damage. In addition, negative linear regressions between lignin and S monomer contents and S/G ratios could be helpful as an alternative to identify resistance in soybean seeds.

Index terms: *Glycine max* (L.) Merrill, guaiacyl, monolignols, seed quality, syringyl.

RESUMO: Sementes de soja são cruciais para a produção global de alimentos. Vários fatores afetam a qualidade das sementes de soja, entre eles os danos mecânicos, que podem levar à redução do potencial germinativo e diminuição do vigor das plântulas. A presença de lignina no tegumento da semente contribui para a resistência aos danos mecânicos. No entanto, a relação entre a composição monomérica da lignina e o dano mecânico é desconhecida. Para preencher esta lacuna, nós avaliamos os teores de monômeros de hidroxifenil (H), guaiacil (G) e siringil (S) em tegumentos de três cultivares de soja, Doko, IAS-5 e Savana. Os resultados revelaram que a composição monomérica da lignina variou entre cultivares resistentes e suscetível. Os teores dos monômeros G e S foram inversos nas cultivares Doko e Savana, sugerindo que a composição dos monômeros de lignina pode desempenhar um papel crucial na resistência das sementes de soja aos danos mecânicos. Além disso, regressões lineares negativas entre os teores de lignina e monômero S e as relações S/G podem ser uma alternativa para identificar resistência em sementes de soja.

Termos para indexação: *Glycine max* (L.) Merrill, guaiacil, monolignois, qualidade de semente, siringil.

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INTRODUCTION

Brazil's soybean harvest reached a record in 2022, making it the world's largest crop producer (CONAB, 2022); therefore, continuous improvement in crop technology practices is necessary to maintain and increase soybean yields. One critical factor influencing soybean productivity is using seeds with high physical, genetic purity, physiological, and sanitary (Dall'Agnol, 2016; Batista et al., 2022). Several factors can affect the quality of soybean seeds, including climatic conditions and preharvest practices. Implementing appropriate pre- and post-harvest techniques is crucial to maintaining seed quality. Thus, selecting suitable genotypes in breeding programs before seed production and commercialization is essential for success (França-Neto et al., 2000; Costa et al., 2001; Cunha et al., 2009; Terasawa et al., 2009; França-Neto and Krzyzanowski, 2019; Brzezinski et al., 2022).

One factor that can impact the quality of soybean seeds is the lignin content, which can be utilized in breeding programs for selection purposes. Soybean seed is a complex structure composed of three main parts: the coat or tegument, cotyledon, and embryonic axis. The tegument, a dead layer, plays a crucial role in seed quality, protecting it against adverse weather, pests, and mechanical damage (Krzyzanowski et al., 2023). High rainfall and humidity oscillations associated with high temperatures can cause damage to the seed coat and result in significant losses during seed production (França-Neto et al., 2016).

Lignin is primarily present in the seed coat or tegument in soybean seeds. The lignin content in the seed coat plays an essential role in protecting the seed from moisture and mechanical damage (Capeleti et al., 2005a; França-Neto et al., 2016). Mechanical damage to soybean seeds can occur during harvesting and processing, reducing seed quality, vigor, and germination.

Found in the cell walls of plants, lignin provides structural support and rigidity to tissues, making them more resilient to various environmental stresses. In general, lignin is essential for plant growth and development because it provides vascular impermeability, resistance to water loss, mechanical strength for growth, and resistance to pathogen attack. Specifically, lignin can be found in the stem tissue, the xylem's tracheal elements, the fiber-filled scleral cells, the vascular tissue, the endoderm cells, the ectoderm cells, and the seed coat cells (Barros et al., 2015). Interestingly, optical and scanning microscopy revealed lignin deposits in the testa of soybean seeds and palisade cell walls (Menezes et al., 2009). In this way, soybean genotypes with higher lignin content in the tegument have better physical and physiological qualities and greater resistance to mechanical damage (Krzyzanowski et al., 2023). Consistent with this, a relevant study using the pendulum test (Carbonell and Krzyzanowski, 1995) showed a direct relationship between seed coat lignin and the resistance index, classifying 12 field-grown soybean cultivars as resistant, moderately resistant, and susceptible to mechanical damage. This dataset suggests a total lignin content higher than 5% indicates soybean seed resistance to mechanical damage (Alvarez et al., 1997).

Structurally, lignin is a nonlinear, heterogeneous biopolymer of simple phenolic acids. Lignin biosynthesis starts with phenylalanine and tyrosine formed in the shikimic pathway, and it involves sequential hydroxylation and methylation of aromatic rings and side-chain modification to produce lignin monomers. This pathway, known as the phenylpropanoid pathway, begins with the deamination of L-phenylalanine to form *t*-cinnamate by the action of phenylalanine ammonia-lyase. Hydroxylation, methoxylation, esterification, and reduction reactions converted *p*-coumaric, caffeic, and ferulic acids to their respective monolignols, i.e., *p*-coumaryl, coniferyl, and sinapyl alcohols. At the end of the route, these monomers are copolymerized to form *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units through the action of laccase and peroxidase to form lignin (Marchiosi et al., 2020).

Over the past five years, the Laboratory of Plant Biochemistry (Bioplan, UEM), in collaboration with several seed production Brazilian companies, has analyzed more than 800 new soybean seed cultivars, many of which still need to be commercialized, intending to evaluate the lignin content of the tegument. Companies are clearly interested in selecting soybean seeds with high lignin content in the soybean seed coat and its direct correlation to mechanical damage resistance. While the contribution of soybean seed coat lignin to resistance to mechanical damage is evident, the role of lignin monomeric composition is unknown. Because the monomeric composition of lignin can vary significantly between different plant tissues (Vanholme et al., 2010), we hypothesized that the contents of typical lignin monomers (H, G, and S) might vary in the seed coats of soybean cultivars, thereby affecting their effect on mechanical damage resistance. To test this hypothesis, the levels of total lignin and its monomeric composition were determined in the seed coats of two resistant (Doko and IAS-5) and one susceptible (Savana) cultivars. Regression analyses between lignin and monomer levels or S/G ratios were assessed as possible indicators of soybean seed resistance to mechanical damage.

MATERIAL AND METHODS

The research was conducted at the Plant Biochemistry Laboratory of the *Universidade Estadual de Maringá* (UEM) in Paraná, Brazil, and at the Brazilian Agricultural Research Corporation (Embrapa Soybean) in Londrina, PR, Brazil. Soybean seed cultivars Doko and IAS-5 (resistant to mechanical damage) and Savana (susceptible to mechanical damage) were selected based on their gravimetric lignin content (Alvarez et al., 1997).

The soybean seeds were soaked in water for 12 hours; the seed coats were removed, dried in an oven for 24 hours, and then ground. Next, 300 mg of the tegument was placed in centrifuge tubes. The cell wall extraction procedure was conducted as described by Moreira-Vilar et al. (2014). First, 7 mL of phosphate buffer was added to stabilize the pH. After vortexing, the material was centrifuged (3300×g for 6 minutes), and the supernatant was discarded. The shaking and centrifugation process was repeated twice, performing each step three times with 7 mL of Triton X100 and twice with 7 mL of NaCl and 7 mL of water. Finally, the addition of 5 mL of acetone precipitates the samples. After this process, the tubes were placed in a vacuum desiccator to flocculate, and then the material was dried in an oven at 60 °C. After drying, the samples were macerated and considered free of interfering proteins. In the next step, 20 mg was weighed and placed into glass tubes. After heating in a water bath (70 °C for 30 minutes), 0.5 mL of 25% acetyl bromide in acetic acid was added to dissolve the lignin. The samples were placed on ice, and 0.9 mL of NaOH was added to stop the reaction. Then, 0.1 mL of hydroxylamine was added. After centrifugation (4000×g for 5 minutes), 0.3 mL of the supernatant was collected and diluted in 2.7 mL of acetic acid. Absorbance was measured at 280 nm, and the results were expressed as g% lignin based on an appropriate standard curve.

The nitrobenzene oxidation method was employed, according to Scalbert et al. (1986), with modifications. The protein-free cell wall (50 mg) was placed in stainless steel bowls with a screw cap containing 0.9 mL of 2 M NaOH and 100 µL of nitrobenzene. The samples were placed in an oven heated to 170 °C for 150 minutes, stirring halfway through the reaction time. After oxidation, the sample was cooled and washed twice with 2 mL of deionized water, and the solution was transferred into a separatory funnel. Then, the sample was rewashed with chloroform. Two washes with chloroform (5 mL per wash) were acidified with 350 µL of 5 M HCl and extracted twice with chloroform (5 mL per extraction). The organic extracts were dried and resuspended in 1 mL of methanol. All samples were filtered through a 0.45 µm filter and analyzed by high-performance liquid chromatography (HPLC). The mobile phase was 4% methanol/acetic acid in water (20/80, v/v), with a flow rate of 1.2 $mL.min^{-1}$ for an isocratic analysis of 20 minutes. Quantifications of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomers were performed at 290 nm using the corresponding standards. The results were expressed as µg of monomer per mg^{-1} of the cell wall.

The data are expressed as the mean ± standard error of four (lignin) and five (lignin monomers) replications. A one-way analysis of variance (ANOVA) was performed to test the significance of differences using the GraphPad Prism® software package (GraphPad Software Inc., San Diego, CA, USA). Data from Figures 4 and 6 were subjected to linear regressions. Tukey's multiple comparison tests were used to compare means, and statistical significance was defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

Figure 1 shows that varieties Doko, IAS-5, and Savana contain 5.0 g%, 4.32 g%, and 3.77 g% lignin, respectively. Quantified by the acetyl bromide method, the lignin contents confirm the resistance of the seeds of the Doko

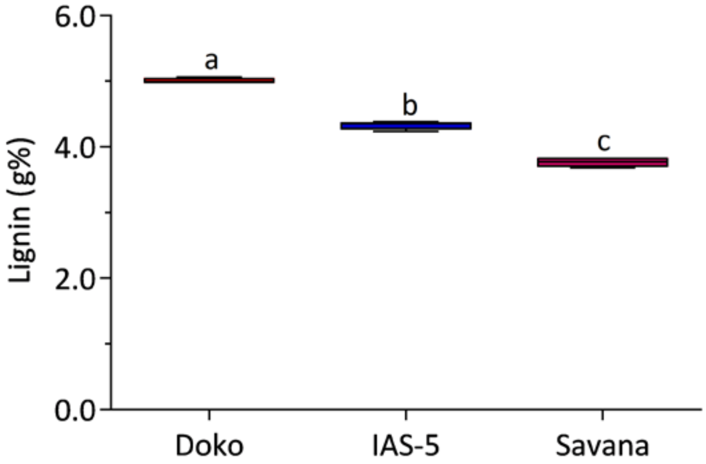

Figure 1. Lignin levels in the seed coats of Doko, IAS-5, and Savana cultivars. Means (n = 4 ± standard error of the mean) marked with different letters are significantly different according to Tukey's multiple comparison test ($p \leq 0.0001$).

and IAS-5 cultivars and the susceptibility of the Savana cultivar to mechanical damage. In fact, these results are consistent with those obtained using the gravimetric lignin method. By this method, seeds of soybean cultivars are classified as resistant (Doko), moderately resistant (IAS-5), or susceptible (Savana) to mechanical damage. However, high-quality seeds like IAS-5 containing at least 4% lignin can also exhibit increased mechanical resistance (Alvarez et al., 1997). These data were also consistent with the classification of these cultivars for seed resistance or susceptibility to mechanical damage when lignin was measured spectrophotometrically using thioglycolic acid (Capeleti et al., 2005b).

The lignin component in soybean seed coats consists of three monomers: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomers (Marchiosi et al., 2020). The monomeric composition of lignin may vary significantly between different plant tissues and varieties, which may affect their resistance to mechanical damage. In addition to canonical monomers (H, G, S), lignin can also contain phenolic compounds derived from the phenylpropanoid pathway in its structure. Among these compounds are lignin monoesters, phenolic compounds derived from the simplified biosynthesis of lignin monoesters, ferulic acid esters, flavonoids, hydroxystilbene, trioctyl, and hydroxycinnamic amides (Del Río et al., 2020). Therefore, accurately quantifying H, G, and S monomers is critical to understanding their roles in soybean seed resistance to mechanical damage.

In the Doko, IAS-5, and Savana cultivars, the H monomer contents were 0.30, 0.35, and 0.26 $\mu g.mg^{-1}$ of tegument, respectively (Figure 2A); the G monomer contents were 0.52, 0.45, and 0.49 $\mu g.mg^{-1}$ of tegument (Figure 2B); and the S monomer contents were 0.16, 0.20, and 0.26 $\mu g.mg^{-1}$ of tegument (Figure 2C). The S/G ratios were significantly different among the three cultivars: 0.31 (Doko), 0.43 (IAS-5), and 0.54 (Savana) $\mu g.mg^{-1}$ of tegument (Figure 2D). Due to its structural complexity, the monomeric composition of lignin varies between plant species (Vanholme et al., 2010). So, two aspects regarding the lignin monomers of Doko (resistant) and Savana (susceptible) cultivars deserve attention. First, the contents of G and S monomers were inverse in the cultivars Doko (higher G content and lower S) and Savana (lower G content and higher S) (Figures 2B and 2C). Second, the composition of G and S monomers generated an inverse S/G ratio between both cultivars (Figure 2D). It is essential to highlight that no study has recently described the monomeric composition of lignin in soybean seed coats. However, the data revealed an exciting difference between one resistant cultivar (Doko) and one susceptible cultivar (Savana).

The main finding of our study was the observed linear regressions between the contents of total lignin (Figure 1) and S monomer (Figure 2C) or S/G ratios (Figure 2D). Both conditions showed negative linear regressions. The ratios between lignin and S monomers were 31.2 (Doko), 21.5 (IAS-5), and 14.6 (Savana) (Figure 3A). Similarly, there was a linear regression between lignin contents and S/G ratios with values of 16.1 (Doko), 10.0 (IAS-5), and 7.0 (Savana) (Figure 3B).

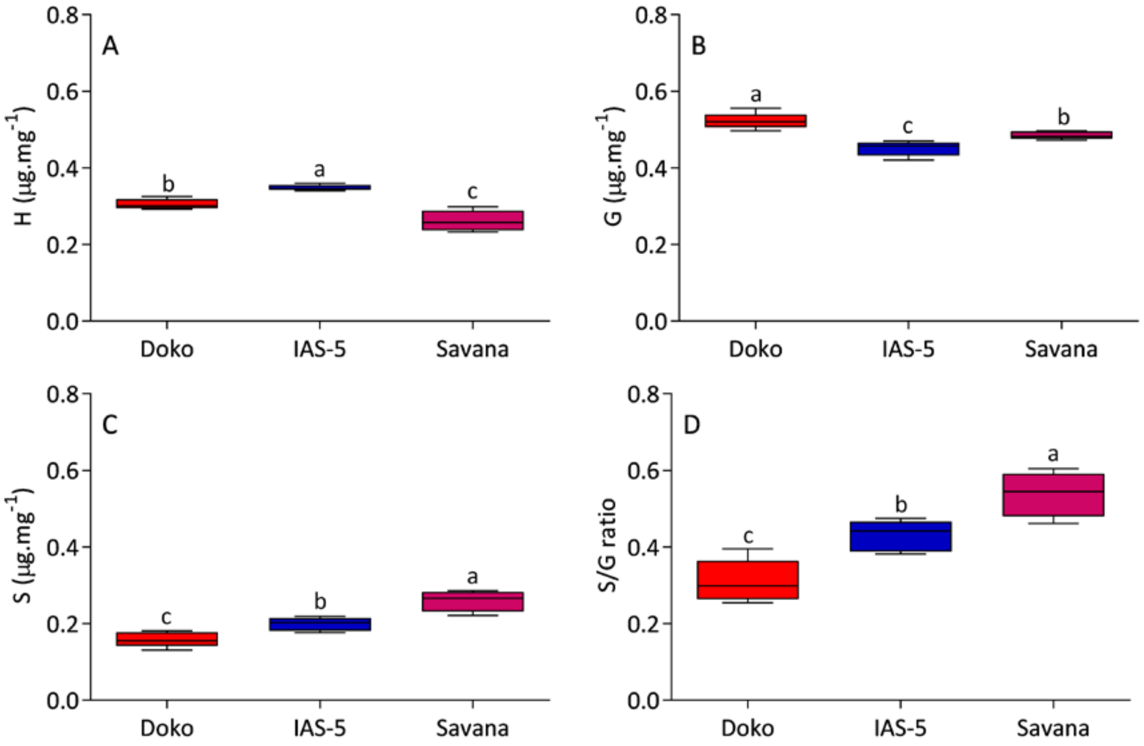

Figure 2. Monomeric composition of lignin in seed coats of Doko, IAS-5, and Savana cultivars. (A), H monomer; (B), G monomer; (C), S monomer, and (D) S/G ratios. Means (n = 5 ± standard error of the mean) marked with different letters are significantly different according to Tukey's comparison test. H (Doko vs IAS-5, $p = 0.0128$; Doko vs Savana, $p = 0.0139$; IAS-5 vs Savana, $p \leq 0.0001$). G (Doko vs IAS-5, $p = 0.0001$; Doko vs Savana, $p = 0.0166$; IAS-5 vs Savana, $p = 0.0248$). S (Doko vs IAS-5, $p = 0.0334$; Doko vs Savana, $p \leq 0.0001$; IAS-5 vs Savana, $p = 0.0037$). S/G ratios (Doko vs IAS-5, $p = 0.0102$; Doko vs Savana, $p = 0.0001$; IAS-5 vs Savana, $p = 0.0238$).

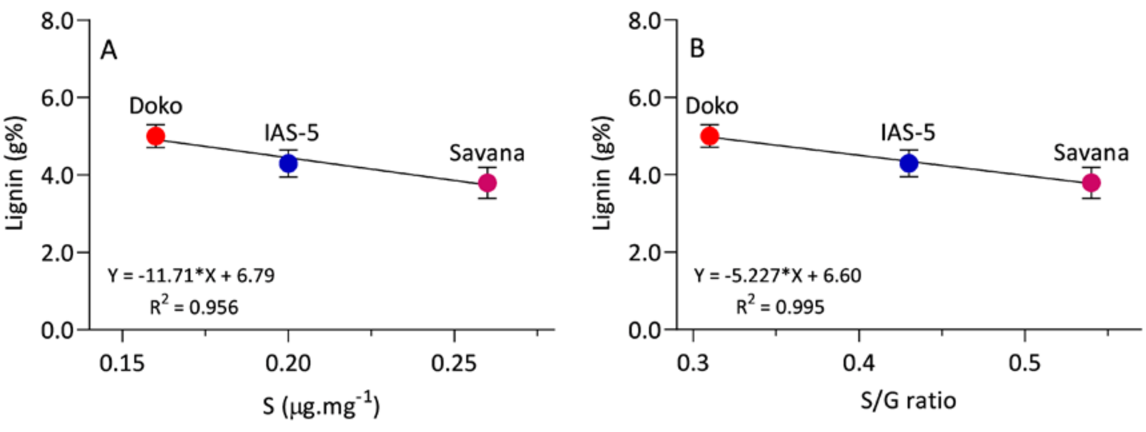

Figure 3. Regression analyzes between lignin levels in seed coats and levels of S monomers (A) or S/G ratios (B) of Doko, IAS-5, and Savana cultivars.

Although studies on the monomeric composition of lignin have not been described in soybean seed coats, some works point to the importance of S/G ratios. At the structural level, S and G units covalently form the backbone of lignin polymers via arylglycerol-β-aryl ether (β-O-4) linkages. The S and G units covalently link together through β-β, 5-5, and β-5 carbon-carbon bonds (Yoo et al., 2020). The S-rich lignin is mainly composed of β-O-4 bonds, with a lower degree of condensation and structural complexity, while G-rich lignin is composed of β-5 and 5-5/4-O-β-bonds, with a higher degree of condensation leading to more branching structures (Ralph et al., 2019). In this context, G-rich lignin with a lower S/G ratio, such as the Doko cultivar, may favor the resistance of its seeds to mechanical damage. On the other hand, S-rich lignin, and a high S/G ratio, as in the case of the Savana cultivar, may indicate its seeds' susceptibility to mechanical damage. Increasing the S/G ratio would result in easily cleaved lignin due to a lower degree of polymerization and, therefore, greater structural linearity of the lignin with less cross-linking. In contrast, lignin with a higher content of G units would have a positive effect on mechanical damage. Thus, the findings suggest that soybean genotypes with higher lignin content in the seed coat and a higher proportion of G units may have better resistance to mechanical damage. The strengthening of this hypothesis involves complementary studies evaluating the monomeric compositions of lignin in the seed coats of other soybean cultivars previously classified in terms of resistance or not to mechanical damage.

CONCLUSIONS

The contents of G and S monomers were shown to be inverse in the Doko and Savana cultivars, indicating that the lignin monomeric composition could play a crucial role in the resistance of soybean seeds.

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