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Lignin content in seed coats of Glyphosate-resistant soybean and their respective parents

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The large-scale cultivation of Roundup Ready (RR) has recently increased among Brazilian farmers. However, few studies have compared the intrinsic characteristics of the seeds of RR soybean cultivars and their respective conventional parental. Thus, the purpose of this study is to verify if the genetic modification of the RR soybean affects the lignin content and its monomeric composition in the seed coats, in comparison with the parental cultivar. To do that, five groups, each one with a RR cultivar and its respective parental were selected. After the physiological analysis of the seeds, the coats were separated and dried in an incubator at 80°C for 24 h. Next, the contents of lignin and its monomers *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) were determined. In order to compare the RR cultivars and their respective parents, a statistical evaluation of contrasts was performed. The results revealed that only the group BRS133 *vs* BRS245RR was different; the transgenic cultivar showed significant increases in the lignin contents and their monomers. In conclusion, the introduction of the sequence CP4-EPSPS in the genome of soybean cultivars, had no any influence on seed coat lignification.

Key words: Monolignols, seeds, *Glycine max* L. Merrill, Roundup®Ready.

INTRODUCTION

The development of the transgenic soybean, resistant to the Roundup Ready© (RR) herbicide, changed the international soybean market forever. Its approval for cultivation was obtained in Brazil in 2005. According to the CONAB (2013), the total acreage for the cultivation of soybean at the 2012/2013 crop reached 27.65 million hectares, with the RR soybean planted in 90% of this area, which is similar to the numbers in the United States and Argentina (Céleres, 2014).

The RR soybean encodes a variation of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (EPSP synthase), which shows a low affinity for glyphosate, creating resistance against this herbicide in this plant. Thus, the enzyme activity remains independent from the presence, or absence, of the glyphosate (Shan et al., 1986; Padgette et al., 1995; Harrison et al., 1996).

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The 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase is an important enzyme of the shikimate pathway, which is responsible for the synthesis of aromatic amino acids, such as phenylalanine, tyrosine and tryptophan. Besides being important for the synthesis of proteins in plants, these amino acids participate in the phenylpropanoid pathway, which is the main route for the synthesis of phenolics, including the monolignols, which are precursors of the lignin polymer. Thus, an inhibition of EPSP synthase by glyphosate can affect not only the production of soybean proteins, but also the phenylpropanoid pathway and, by consequence, the lignin production. Lignin is associated with many different specialized cells to fulfil specific physiological functions, but exhibits distinct properties for each cell type, which may explain why no general mechanism for lignification has been yet defined (Barros et al., 2015).

At the cytosol, the pathway begins with the phenylalanine ammonia lyase (PAL), which deaminates the L-phenylalanine in *t*-cinnamic acid. The second step is the hydroxylation of the *t*-cinnamic acid by the cinnamate 4-hydroxylase (C4H), generating *p*-coumaric acid (first phenylpropanoid in the free acid form). Next, a hydroxylation occurs on the position 3 of the *p*-coumaric acid by the *p*-coumarate 3-hydroxylase (C3H), producing the caffeic, ferulic, 5-hydroxyferulic and sinapic acids, respectively. The free acid forms of the phenylpropanoids are connected to a coenzyme A through the action of the 4-coumarate: coenzyme A ligase (4-CL), reduced to the aldehydic forms by the cinnamoyl-CoA reductase (CCR) and, next, reduced to the alcoholic forms through the action of the cinnamyl alcohol dehydrogenase (CAD) (Boerjan et al., 2003). Finally, the *p*-coumaryl, coniferyl and sinapyl alcohols (or monolignols) are transported to the apoplast (Escamilla-Trevino et al., 2006). In the cell wall, these monolignols are converted into the respective monomers *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), which are polymerized into lignin by action of peroxidases and laccases (Bray et al., 2000).

It is well known that the content of lignin may vary depending on plant species, development stage and tissue type. For example, the high lignin content in the stalk of eucalyptus (*Eucalyptus* sp) is damaging to the extraction of paper and cellulose pulp (Endt et al., 2000). The high content of lignin at the upper part of a fodder causes low digestibility and, consequently, bad pastures (Lacerda et al, 2003).

Lignin deposition depends on the cell type, the developmental stage and the species. This spatial distribution is characterized by differences in time, amount, size and monomeric composition of the lignin polymer (Terashima et al., 2012). Some studies have revealed different lignin contents between RR and conventional plants, which can be due to the intense lignification in transgenic cultivars. An enhanced production of lignin of up to 20% more in transgenic cultivars has been related (Coghlan, 1999; Kuiper et al., 2001). Under water deficit and high temperatures, the overproduction of lignin causes fissures and breakages in the soybean stems, as noted in United States and Brazil (Nodari and Destro, 2002). Based on these reports, the hypothesis that claims that RR soybean cultivars contain higher contents of lignin, when compared to the conventional cultivars, has gained strength. It is due to the fact that the sequence CP4-EPDPS inserted in the genome of soybean to produce a glyphosate-resistant plant may cause a pleiotropic effect and, thus, modify the synthesis of lignin. In this context, the present study intends to determine the content of lignin and its monomeric composition in the seed coats of five RR soybean and their respective conventional cultivars.

MATERIALS AND METHODS

Five soybean cultivars were selected RR (BRS 245RR, BRS 255RR, BRS 242RR, Emgopa 33RR and Emgopa 316RR) and their respective conventional parental cultivars (BRS 133, BRS 137, Embrapa 48, Emgopa 313 and Emgopa 316). Initially, the seeds were submitted to germination tests (Brasil, 2009) in order to evaluate its quality. The soybean cultivars used in the experiment were grown in the city of Rio Verde, GO (17°47'24'' S; 50°56'31'' W; a 740 m altitude). The predominant climate is Cwa, according to the Köppen classification and the soil used was a typic dystrophic Red Oxisol, medium texture.

A sample of 200 seeds of each cultivar was submerged in water for 12 h. After this period, the coats were manually separated from the seeds, dried in an incubator (80°C, 16 h) and grinded. To determine the lignin content, dry coat (0.3 g), was homogenized in 50 mM phosphate buffer pH 7.0 (7 ml) and transferred to a centrifuge tube (Ferrarese et al., 2002). The precipitate was centrifuged (1.400*g*, 6 min.), washed and successively centrifuged, as follows: twice with 50 mM phosphate buffer pH 7.0 (7 ml); 3 times with 1% (v/v) Triton® X-100 in buffer pH 7,0 (7 ml); twice with 1 M NaCl in buffer pH 7.0 (7 ml); twice with distilled water (7 ml) and twice with acetone (5 ml). The material was dried in an incubator (80°C, 24 h) and the resulting sample was defined as a fraction of the cell wall free of proteins. Next, the sample was used to determine the total content of lignin through the acetyl bromide method (Morrison, 1972). A portion (20 mg) of the sample was placed in a centrifuge tube with 500 µl of acetyl bromide at 25%. The samples were heated (70°C, 30 min.), transferred to an ice bath and the reaction was interrupted by the addition of 0.9 ml of NaOH 2 M. Then, 0.1 ml of hydroxylamine HCl 7.5 M and 2 ml of cold acetic acid were added. The samples were centrifuged (1.000 g, 5 min.), and the supernatant was diluted and the reading were taken at 280 nm. The lignin content was determined according to a standard curve and recorded in mg lignin g^{-1} of the cell wall.

In order to determine the monomeric composition of the lignin, the nitrobenzene oxidation method was performed. A protein-free fraction of the wall cell (50 mg) was placed in a Pyrex® ampoule containing 1 ml of NaOH 2 M and 100 µl of nitrobenzene. The ampoule was sealed and heated (170°C, 150 min.), while shaking the sample occasionally during the reaction. After oxidation, the sample was cooled, washed twice with chloroform, acidified with 350 µl of HCl 5 M, and extracted twice with chloroform. The organic extracts were combined, dried and re-suspended in methanol. All samples were filtered through a 0.45-µm disposable syringe filter and analyzed (20 µl) with a Shimadzu® Liquid Chromatograph equipped with an LC-10AD pump, a Rheodyne® injector, an SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack®

Table 1. Lignin contents and its monomeric composition in glyphosate-resistant soybean seeds coats and their respective conventional cultivars.

Lignin monomer					
Cultivar	**Lignin**	**H**	**G**	**S**	**H + G + S**
BRS 133	54.12±0.00[a]	0.02±0.003[a]	0.10±0.014[a]	0.01±0.003[a]	0.13±0.018[a]
BRS 245RR	71.80±0.00[b]	0.06±0.004[b]	0.17±0.004[b]	0.02±0.002[a]	0.26±0.010[b]
BRS 137	67.46±0.00[a]	0.08±0.004[a]	0.18±0.001[a]	0.04±0.003[a]	0.32±0.019[a]
BRS 255RR	63.85±0.00[a]	0.06±0.005[a]	0.22±0.010[a]	0.03±0.004[a]	0.31±0.017[a]
Embrapa 48	67.72±0.00[a]	0.08±0.006[a]	0.15±0.002[a]	0.02±0.002[a]	0.26±0.009[a]
BRS 242RR	66.74±0.00[a]	0.07±0.005[a]	0.17±0.001[a]	0.03±0.007[a]	0.28±0.020[a]
Emgopa 313	67.65±0.00[a]	0.06±0.004[a]	0.23±0.014[a]	0.06±0.005[a]	0.34±0.023[a]
Emgopa 313RR	71.13±0.00[a]	0.08±0.004[b]	0.30±0.025[b]	0.08±0.008[b]	0.46±0.038[b]
Emgopa 316	53.22±0.00[a]	0.06±0.004[a]	0.13±0.007[a]	0.03±0.003[a]	0.22±0.014[a]
Emgopa 316RR	53.61±0.00[a]	0.07±0.004[a]	0.14±0.006[a]	0.04±0.002[a]	0.25±0.015[a]

The results are expressed in mg g^{-1} of cell wall (for lignin) and µg mg^{-1} of cell wall (for the monomers). Means followed by the same letter, within contrasts, do not differ significantly through the F test, respectively, at 5% probability. H, monomer *p*-hydroxyphenyl; G, monomer guaiacyl and S, monomer syringyl.

CLC-ODS (M) column (150×4.6 mm, 5 µm) was used at room temperature together with the same type of pre-column (10×4.6 mm). The mobile phase was methanol/acetic acid 4% in water (20/80, v/v), with a flow of 1.2 ml min^{-1} for isocratic race of 20 min. The quantification of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde occurred at 290 nm using the corresponding standards. The results were expressed as µg monomer mg^{-1} of wall cell.

The experimental design used was entirely randomized, with 4 repetitions performed for each type of evaluation. Then, the data were submitted to the variance analysis, and compared the means of the transgenic cultivars and their respective parents with the F test ($p > 0.05$) and the option "contrast" of the SAS software.

RESULTS AND DISCUSSION

The comparison between RR cultivars and their respective parents indicated a significant difference (F test at 5% P) only for the contrast formed by the cultivars BRS 133 and BRS 245RR (Table 1). An increase of 33% in the lignin content was noted in the RR cultivar, when compared with the conventional cultivar.

Some studies suggest that RR soybean plants, which have suffered a physiological stress during certain period of times are more lignified. Nodari and Destro (2002) evaluated 9 Brazilian soybean crops during a period of drought and high temperatures, and they observed that RR cultivars contained fissures, bents or breaks (about 50 to 70% of the plants), which were probably due to an enhanced lignin production. According to Coghlan (1999) under high temperature (45°C, for example), the high lignin content hardens and breaks the soybean stems. A similar behavior occurred in United States crops, with great loss of productivity (Nodari and Destro, 2002). Investigating the lignin contents in seed coats of 5 different contrasts formed by conventional and RR soybean, Gris et al. (2010), noted significant difference ($p<0.05$) only in the Jataí *vs* Silvânia RR contrast. The transgenic cultivar contained 27% more lignin than the conventional cultivar.

As noted herein, except for one contrast, the lignin contents were not found in others cultivars (Table 1). It can be due to the possible interferences in the quantification of this polymer, or still, the fact that the RR parentage was attained through backcrossing and, therefore, is not completely isogenic to its parent strains. Another factor refers to the structural complexity of the lignin molecule; a challenge for its quantification in different tissues. This occurs because the most of the techniques used for this purpose are carried out at 280 nm. It is well known that proteins and aromatic compounds absorb energy in this spectral range and, therefore, the presence of these substances may interfere in the quantification of lignin (Vilar et al., 2014). It is important to point out that several authors have quantified lignin by gravimetric analysis (Ferreira, 2003). However, the applied methodology in this work to determine lignin removes the interference of proteins, and allows a more precise analysis (Capeleti et al., 2005).

The obtained results herein suggest a different behavior only for the BRS 133 *vs* BRS 245RR contrast referring to the content of lignin in the soybean's seed coats. However, this finding is not enough to state that the insertion of the CP4-EPSPS sequence in the soybean

genome increases the seed coats lignification. Nevertheless, possible structural changes may occur in this polymer. It can be due to the fact that the insertion of the gene that causes resistance against glyphosate in the RR soybean takes place in the same pathway of the lignin. Thus, further experiments were conducted to quantify the lignin monomers: H, G and S (Table 1).

Similarly to the contents of lignin, the BRS 133 *vs* BRS 245RR contrast showed also significant differences in the composition of H and G monomers (Table 1). In the transgenic cultivar (BRS 245RR), the contents of the H and S monomers were, respectively, 200 and 70% higher than the conventional cultivar (BRS 133). This findings corresponds to an increase of 100% when the lignin content is referred as the sum H+G+S.

Although the lignin contents were similar in the Emgopa 313 *vs* Emgopa 313RR contrast, the monomeric composition was different (Table 1). In fact, the contents of H, G and S monomers were higher in the transgenic cultivar (on average 32%) in comparison with the conventional cultivar. This result corresponds to an increase of 35% of lignin content, referred to as the sum H+G+S.

Conclusion

The obtained results indicated a different behavior in certain RR soybean cultivars in regards to the lignin content and its monomeric composition in the seed coats. However, the differences were significant only for one of the evaluated contrasts. Thus, it is possible to conclude that the CP4 EPSPS sequence, introduced in the genome of soybean cultivars, had no influence on the lignification process of the seed coats.

Conflict of Interests

The authors have not declared any conflict of interests.

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