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Growth and root lignification of susceptible and glyphosate-resistant soybean

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ABSTRACT. Glyphosate resistance is conferred to soybean (*Glycine max* L. Merril) by incorporating a gene encoding a glyphosate-insensitive enzyme (CP4-EPSP synthase) that acts in the shikimate/chorismate pathway, an important metabolic route in the lignification process. The aim of this work was to investigate the root growth and lignin contents of susceptible (CD 201 and OC 14) and glyphosate-resistant (CD 214RR and CD 213RR) soybean cultivars. To this end, three-day-old seedlings were cultivated in half-strength Hoagland nutrient solution (pH 6.0) in a growth chamber (25°C, 12-h photoperiod, irradiance of 280 μmol m^{-2} s^{-1}) for 24 to 96 hours. The results revealed that glyphosate-resistant (CD 213RR and CD 214RR) cultivars showed high root growth when compared to the conventional (OC-14 and CD 201) cultivars. CD 213RR showed high root lignin content and reduced root weight compared to the conventional (OC 14) cultivar, although CD 214RR and CD 201 did not follow the same trend. Based on these results, it is possible to conclude that (1) the different form of EPSP synthase encoded in RR soybean may interfere in phenylpropanoid pathway and further in lignin biosynthesis, and (2) other genetic characteristics inherent to each cultivar may affect roots lignin content in soybean seedlings since lignification in CD 214 RR was not affected of similar manner than cultivar CD 213 RR.

Keywords: glyphosate-resistant soybean, lignin, root growth, soybean.

RESUMO. Crescimento e lignificação de raízes de soja suscetível e resistente ao glifosato. A resistência ao glifosato é conferida à soja (*Glycine max* L. Merrill) pela incorporação de um gene que codifica a enzima CP4-EPSP sintase, uma variante da EPSP sintase, insensível ao glifosato, que atua na via do chiquimato/corismato, importante rota metabólica envolvida na lignificação. O objetivo deste trabalho foi o de investigar o crescimento e os teores de lignina nas raízes de cultivares de soja, suscetíveis (CD 201 e OC 14) e resistentes (CD 214RR e CD 213RR) ao glifosato. Para isso, plântulas com três dias de desenvolvimento foram cultivadas em solução nutritiva de Hoagland, meia-força (pH 6,0), em câmara de crescimento (25°C, fotoperíodo de 12 h, irradiância de 280 μmol m^{-2} s^{-1}) de 24 a 96h. Os resultados revelaram que as cultivares resistentes ao glifosato (CD 213RR e CD 214RR) apresentaram elevado crescimento das raízes quando comparadas com as cultivares convencionais (OC-14 e CD 201). A cultivar CD 213RR apresentou altos teores de lignina e reduzido crescimento das raízes em comparação com a cultivar convencional (OC 14). O mesmo não foi observado nas cultivares CD 214RR e CD 201. Com base nos resultados, é possível concluir que (1) uma forma diferente de EPSP sintase pode interferir na via de fenilpropanoides e, posteriormente, na síntese de lignina, e (2) outras características genéticas inerentes a cada cultivar pode afetar os conteúdos de lignina nas raízes, haja vista que a lignificação na cultivar CD 214RR não foi afetada de similar maneira que na cultivar CD 213RR.

Palavras-chave: soja resistente ao glifosato, lignina, crescimento de raízes, soja.

Introduction

Development of herbicide-resistant cultivars has become an important alternative for weed control in crop systems. Genetic modification of plants with a gene encoding glyphosate (Roundup® Ready active ingredient) tolerance has been done since the 1980s (PADGETTE et al., 1995). Among several species, soybean (*Glycine max* L. Merrill) has turned the attention of researchers due to its relevant economical importance. Roundup® acts on several plant physiological events, reducing chlorophyll and protein synthesis and enhancing ethylene production (GALLI; MONTEZUNNA, 2005). The active ingredient, glyphosate (*N*-phosphonomethyl glycine), inhibits a

specific enzyme of the metabolism of aromatic amino acids, 5-enolpyruvylshikimate-3-phosphate-synthase (EPSP synthase). This enzyme acts in the shikimate pathway, which turns carbohydrates precursors derived from glycolysis and pentose phosphate pathway to aromatic amino acids (tryptophan, tyrosine and phenylalanine) and other ring-containing metabolites (DELANNAY et al., 1995). By inhibiting EPSP synthase, glyphosate blocks the reaction that converts 5-phosphoshikimate to 3-enolpyruvyl-5-phosphoshikimate (PADGETTE et al., 1995). This inhibition reduces the biosynthesis of aromatic amino acids, which leads to several metabolic disturbances, including the arrest interruption of protein production, prevention of secondary product formation, and general metabolic disruption followed by death.

Glyphosate resistance is conferred in soybean by incorporating the gene which encodes a glyphosate-insensitive EPSP synthase (CP4-EPSP synthase). This enzyme, when present, allows the soybean to bypass glyphosate-inhibited native EPSP synthase in the shikimate pathway, thus preventing aromatic amino acids starvation and deregulation of this metabolic route, both of which follow glyphosate treatment in susceptible plants. Thereby, the glyphosate-resistant soybean remains unaffected when treated with the herbicide (HARRISON et al., 1996).

Linked to the shikimate pathway, the phenylpropanoid biosynthetic pathway is also an important metabolic route due to its role in the synthesis of phenolic compounds and a wide range of secondary plant products, including lignin (BOERJAN et al., 2003). Lignin is conventionally defined as a complex hydrophobic network of phenylpropanoid units derived from the oxidative polymerization of one or more of three types of hydroxycinnamyl precursor alcohols (*p*-coumaryl, coniferyl and sinapyl alcohols), which are derived from aromatic amino acid phenylalanine (DEAN, 1997; SEDEROFF et al., 1999). Lignin contributes to the compression strength of stems, imparts mechanical support and allows for the efficient conduction of water and solutes over long distances within the vascular systems and defense in vascular plants (BOERJAN et al., 2003; BOUDET, 2000; BOUDET et al., 2003). Furthermore, quantification of lignin in seeds is important for soybean breeding programs, as it is related to seed mechanical damages (KRZYZANOWSKI, 1998) and storage (KRZYZANOWSKI et al., 2008).

Since lignification is important for the plant growth, it is interesting to evaluate whether the genetic modification in glyphosate-resistant soybean affects its lignin level, by providing information about possible side-effects due to this change. Thus, the aim of the present work was to investigate the root growth and lignin contents of susceptible (CD 201 and OC 14) and resistant (CD 214RR and CD 213RR) soybean cultivars.

Material and methods

Seeds of the soybean (*Glycine max* L. Merr.) cultivars (CD 201 and OC 14 – susceptible – and CD 213RR and CD 214RR – glyphosate resistant) were supplied by COODETEC (Cascavel, Paraná State, Brazil).

Seeds, surface-sterilized with 2% sodium hypochlorite for 3 min and rinsed extensively with deionized water, were dark-germinated (at 25°C) on three sheets of moistened filter paper. Twenty-five 3-day-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred into a glass container (10 x 16 cm) filled with 200 mL of half-strength Hoagland's solution. This nutrient solution was buffered with 17 mM potassium buffer, adjusted to pH 6.0 and monitored over time. The container was kept in a growth chamber (25°C, 12/12 hours light dark^{-1} photoperiod, irradiance of 280 μmol m^{-2} s^{-1}). Roots were measured at the beginning and at the end of experiments (from 24 to 96 hours). Immediately after incubation, the roots were carefully blotted with an absorbent paper and the fresh weight was determined. Dry root weight was estimated after oven-drying at 80°C until it reached a constant weight. Reagents used were of the purest grade available.

After the germination period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 mL, pH 7.0) with a mortar and pestle and transferred to a centrifuge tube (FERRARESE et al., 2002; MARCHIOSI et al., 2009). The pellet was centrifuged (1400*g*, 4 min.) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer pH 7.0 (7 mL); three times with 1% (v v^{-1}) Triton® X-100 in pH 7.0 buffer (7 mL); twice with 1 M NaCl in pH 7.0 buffer (7 mL); twice with distilled water (7 mL); and twice with acetone (5 mL). The pellet was dried in an oven (60°C, 24 hours) and cooled in a vacuum desiccator. The dry matter was defined as a protein-free cell wall fraction. Further, all dry protein-free tissue was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 mL of thioglycolic acid plus 6 mL of 2 M HCl) and heated (95°C, 4 hours). After cooling at room temperature, the sample was centrifuged

(1400*g*, 5 min.) and the supernatant was discarded. The pellet, containing the complex lignin-thioglycolic acid (LTGA), was washed three times with distilled water (7 mL) and the LTGA extracted by shaking (30ºC, 18 hours, 115 oscillations $min.^{-1}$) in 0.5 M NaOH (6 mL). After centrifugation (1400*g*, 5 min.), the supernatant was stored. The pellet was washed again with 0.5 M NaOH (3 mL) and mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (1.8 mL). After precipitation (0°C, 4 hours), LTGA was recovered by centrifugation (1400*g*, 5 min.) and washed twice with distilled water (7 mL). The pellet was dried at 60°C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm. Lignin was expressed as mg LTGA g^{-1} dry weight.

The experimental design was completely randomized in a 4 x 4 factorial (4 cultivars x 4 incubation times) with 4 samples. Each plot was represented by one glass container with twenty-five seedlings. The one-way analysis of variance to test the significance of the observed differences was performed with the SAS package. The means of glyphosate-resistant and susceptible soybeans, inside contrasts, were compared by F test, using the 'contrast' option of SAS package at 5% of significance. After that, means were compared by Tukey test, and P values ≤ 0.05 were considered to be statistically significant.

Results and discussion

Comparative results between glyphosate-resistant and their respective non-resistant cultivars may be seen on Table 1. Data reveals significant differences in the primary root relative length between CD 214 RR and CD 201, after 48 and 72 hours of incubation. The root length was greater in CD 214RR cultivar than in its susceptible parental (CD 201). However, a similar trend was not observed after 96 hours. Table 1 also shows that the primary root relative lengths were significantly different between CD 213RR and its susceptible parental (OC 14 cultivar), after 72 and 96 hours of incubation.

Similarly to length, the root growth may be evaluated by fresh weight (Table 2). In this case, not only primary root length is considered, but also its dimension and the presence of secondary roots. Comparative analysis revealed no significant differences in root fresh weight between glyphosate-resistant (CD 214RR) and conventional (CD 201) seedlings, at any time of incubation (Table 2). However, significant differences were noted between glyphosate-resistant (CD 213RR) and conventional (OC 14) soybean seedlings grown for 48 and 72 hours. No significant differences in dry weight were observed between both conventional and glyphosate-resistant cultivars after incubation (data not shown). Although no field studies have been done to confirm this fact, it is possible that high lignin content in roots may reduce their growth, impairing shoot development. Coghlan (1999) reported that RR soybean higher lignin content in shoots indicates higher susceptibility to cracking. It can be explained by the fact that, as lignin content enhances, it fills spaces previously required for cellulose, causing shoot rigidity and less flexibility.

In general, glyphosate-resistant cultivars showed greater primary root relative length in the most periods of incubation (Table 1). A lower root fresh weight was observed in CD 213RR, although this difference was not observed after 96 hours. Reduced root development in glyphosate-resistant soybean may be explained by the fact that the incorporation of the CP4-EPSPS gene in this plant species leads to drastic changes in the biosynthetic pathway of aromatic amino acids, reducing their levels (BRENBROOK, 2001). In agreement with this, Padgette et al. (1995) evaluated up to 50 physiological and biochemical features of glyphosate-resistant soybean line 40-3-2RR and its respective parental, including aromatic amino acids. The authors observed significant reduction of the phenylalanine level in RR line. Sidhu et al. (2000) also found differences between the tyrosine levels in RR and conventional lines of maize (*Zea mays* L.), which were reduced in RR line. For the authors, these differences may be due to the incorporation of the resistance gene.

Table 1. Primary root relative length (cm) of seedlings of glyphosate-resistant soybean and respective conventional cultivars in different times of incubation in nutrient solution under 12 hours photoperiod at 25°C. Contrast 1 – CD 214RR and CD 201. Contrast 2 – CD 213RR and OC 14.

Cultivar	Primary root relative length (cm)			
	Time of incubation in nutrient solution			
Contrast 1	24 hours	48 hours	72 hours	96 hours
CD 214RR	2.56 a	5.46 a	7.71 a	8.69 a
CD 201	2.33 a	4.43 b	6.83 b	8.36 a
Contrast 2				
CD 213RR	2.64 a	5,22 a	7.63 b	12.05 a
OC 14	2.74 a	5.43 a	8.58 a	10.08 b
CV=9.63				
DMS=0.57				

Means in each contrast inside columns were compared by Tukey test at 5% of probability.

The presence of CP4-EPSPsynthase gene in CD 214 RR did not condition the development of

shorter and fewer roots than parent cultivar CD 201, which suggests that the difference found in the RR variety from the other group (CD 213 RR) occurred by the influence of other genetic characteristics of this material, not only by the incorporation of CP4-EPSPsynthase gene.

Table 2. Root fresh weights of seedlings of glyphosate-resistant soybean and respective conventional cultivars in different times of incubation in nutrient solution under 12 hours photoperiod at 25°C. Contrast 1 – CD 214RR and CD 201. Contrast 2 – CD 213RR and OC 14.

Cultivar	Fresh weight / seedling (g)			
	Time of incubation in nutrient solution			
Contrast 1	24 hours	48 hours	72 hours	96 hours
CD 214RR	0.0973 a	0.1814 a	0.2409 a	0.3077 a
CD 201	0.0924 a	0.1659 a	0.2530 a	0.3316 a
Contrast 2				
CD 213RR	0.0825 a	0.0992 b	0.1427 b	0.2845 a
OC 14	0.0865 a	0.1947 a	0.1720 a	0.2672 a
CV=9.68				
DMS=0.0167				

Means in each contrast inside columns were compared by Tukey test at 5% of probability.

Although no significant changes occurred in the root lignin content between glyphosate-resistant (CD 214RR) and conventional (CD 201) cultivars, its levels increased in the CD 213RR when compared to the conventional (OC 14) cultivar (Table 3). Contrast analysis between RR and conventional cultivars indicated significant difference only after 72 and 96 hours of incubation. CD 213RR showed high lignin content in roots in these periods.

Table 3. Mean values of root lignin content (mg LTGA g^{-1} root) in seedlings of glyphosate-resistant soybean and respective conventional cultivars in different times of incubation in nutrient solution under 12 hours photoperiod at 25°C. Contrast 1 – CD 214RR and CD 201. Contrast 2 – CD 213RR and OC 14.

Cultivar	Lignin content (mg LTGA g^{-1} root) in root			
	Time of incubation in nutrient solution			
Contrast 1	24 hours	48 hours	72 hours	96 hours
CD 214RR	10.62 a	16.09 a	17.49 a	21.15 a
CD 201	10.42 a	14.82 a	17.87 a	19.59 a
Contrast 2				
CD 213RR	11.49 a	12.96 a	14.79 a	22.45 a
OC 14	9.76 a	12.61 a	12.05 b	15.90 b
CV=8.22				
DMS=1.77				

Means in each contrast inside columns were compared by Tukey test at 5% of probability.

After 96 hours of incubation, lignin content in CD 213RR increased 6.5 mg LTGA g^{-1} root, which was 29.2% higher than the lignin content of its parental (OC 14). Based on these results, it is possible to infer that the high lignin content found in roots of CD 213RR, when compared to the conventional cultivar, may be due to the glyphosate-resistance gene incorporation. Whether this is true, it seems quite plausible to suppose that the expression of a different form of EPSP synthase (PADGETTE et al., 1995) may interfere in the phenylpropanoid pathway, changing lignin biosynthesis. Additionally, studies with other RR cultivars and different times of incubation, and the determination of enzymes related to the metabolic pathway are necessary to strength this hypothesis. This is the challenge of a new study in progress.

Conclusion

Glyphosate-resistant soybean (CD 213RR and CD 214RR) showed greater root length compared with conventional cultivars (OC 14 and CD 201). Expressive data verified in cultivar CD 213RR can be due to a particular genetic characteristic. CD 213RR showed higher root lignin content and reduced root weight until 72 hours of evaluation, when compared to its conventional cultivar (OC 14). These findings suggest that (1) the expression of a different form of EPSP synthase may interfere in the phenylpropanoid pathway and further in the lignin production, and (2) other genetic characteristics inherent to each cultivar may affect roots lignin content in soybean seedlings.

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