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Glyphosate-induced metabolic changes in susceptible and glyphosate-resistant soybean (*Glycine max* L.) roots

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ABSTRACT

Glyphosate (*N*-(phosphonomethyl)glycine) blocks the shikimate pathway, reducing the biosynthesis of aromatic amino acids, followed by the arrest interruption of protein production and a general metabolic disruption of the phenylpropanoid pathway. Glyphosate-resistance is conferred to soybean by incorporating a gene encoding a glyphosate-insensitive enzyme (CP4-EPSP synthase) that acts in the shikimate pathway. This paper evaluates the metabolic effects caused by this herbicide on the shikimate (shikimate dehydrogenase activity and shikimate content) and phenylpropanoid (phenylalanine ammonia-lyase activity, phenolic and lignin contents) pathways in BRS-133 (susceptible) and BRS-245RR (resistant) soybean (*Glycine max* L.) roots. In general, the results showed that in susceptible roots (1) glyphosate affects the shikimate pathway (massive shikimate accumulation and enhanced shikimate dehydrogenase activity) and the phenylpropanoid pathway (increase in PAL activity, production of benzoate derivatives and decrease of lignin) and (2) the metabolic disruption contributes to the production of *p*-hydroxybenzoate and vanillate, which likely originate from shikimate and/or cinnamate and their derivatives. No such changes were observed in the genetically modified soybean consistent with its resistance to glyphosate.

1. Introduction

The shikimate pathway, one of the major biosynthetic pathways in higher plants, consists of seven enzymatic steps and ends with the formation of chorismate, the branch point precursor for the biosynthesis of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. The shikimate pathway is localized in plastids of all plant tissue types, including non-green tissues such as most roots [1]. The third and fourth steps of the pathway are catalyzed by the bifunctional enzyme 3-dehydroquinate dehydratase (DHD, EC 4.2.1.10)/shikimate dehydrogenase (SDH, EC 1.1.1.25), which catalyses the dehydration of 3-dehydroquinate to 3-dehydroshikimate and the reversible reduction of 3-dehydroshikimate to shikimate [2]. Another step is the reversible formation of 5-enolpyruvylshikimate 3-phosphate (EPSP) and inorganic phosphate from shikimate 3-phosphate and phosphoenolpyruvate. This reaction is catalyzed by EPSP synthase (EC 2.5.1.19). Linked to this 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. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), which catalyzes the elimination of ammonia from phenylalanine to provide cinnamate, is located at the starting point of the phenylpropanoid pathway, interfacing with the shikimate pathway and regulating the production of phenolic compounds, monolignol polymerization, and lignin synthesis [3].

In plants, shikimate pathway enzymes respond to different environmental stresses such as heavy metals [4], herbivore and microbial attack, wounding, or nitrogen and amino acid starvation [5]. The shikimate pathway has thus been an attractive target for the design of herbicidal agents. For example, EPSP synthase is the only cellular target for the glyphosate (*N*-(phosphonomethyl)glycine), the most extensively used foliar-applied, broad-spectrum, non-selective herbicide, which is particularly active against annual and perennial plants [6]. The inhibition of EPSP synthase by glyphosate 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 [6]. With the inhibition of EPSP synthase, shikimate, the metabolite upstream of this enzyme in the pathway, cannot be converted into EPSP. Blockage of the shikimate pathway consequently results in the accumulation of high levels of shikimate [7–9]. Since shikimate accumulation is a direct result of herbicide inhibition of EPSP synthase, increased shikimate is a good biomarker for glyphosate exposure [7,8,10–12].

Glyphosate-resistance is conferred in soybean (*Glycine max* L. Merr.) by incorporating a gene which encodes a glyphosate-insensitive EPSP synthase (CP4-EPSP synthase). This enzyme, when

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expressed, 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 (GR) transgenic soybean remains unaffected when treated with the herbicide [7,13]. Glyphosate is readily translocated from leaves to the roots, rhizomes, and apical tissues of treated plants. Its metabolic degradation is very slow and it accumulates in metabolic sinks such as young roots and developing and mature nodules. In fact, a single foliar application of 0.5 kg ha^{-1} of glyphosate caused the accumulation of $\leqslant$0.3 mM in the root tissues of susceptible plants [6]. Thus, the present work evaluates the metabolic effects caused by this herbicide on the shikimate (SDH activity and shikimate content) and phenylpropanoid (PAL activity and phenolic and lignin contents) pathways of BRS-133 (susceptible) and BRS-245RR (GR) soybean roots. For this investigation, early seedling growth was used for the short-term exposure to glyphosate.

2. Materials and methods

2.1. General procedures

Seeds of the soybean (*G. max* L. Merr.) genotypes (BRS-133—susceptible—and BRS-245RR—GR) were supplied by National Soybean Research Center (Embrapa Soybean, Brazil), which previously analyzed the presence of CP4 EPSPS gene in BRS-245RR by using polymerase chain reactions (PCR). Seed BRS-245RR contains 98.44% genetic information of BRS-133 plus the CPS EPSPS gene and corresponds to BRS-133 (conventional soybean).

Seeds (200) of each soybean cultivar were immersed either in 300 mL of 0.1–5 mM glyphosate solution or in water (control) for 4 h. After this short immersion period, the seeds were spread on three layers of germination paper (28 × 38 cm, Germilab®, Brazil) previously moistened with 100 mL of distilled water. The paper sheets were rolled up and placed vertically in plastic containers (11 cm diameter, 36 cm height), containing 50 mL of distilled water. The containers were placed in a germination chamber, and seeds were germinated in darkness. A temperature regime of 25 °C (±0.2) and relative humidity ranging between 70% and 80% was maintained during the germination. Three days later, the roots were excised and analyzed. When indicated, the fresh root weight was determined immediately after germination and the dry weight was estimated after oven-drying at 80 °C until a constant weight was achieved. Glyphosate (*N*-(phosphonomethyl)glycine) 95% purity, and shikimic acid, min 99% purity, were purchased from Sigma (St. Louis, MO), and all other reagents used were of chromatographic grade or of the purest grade available.

2.2. Enzymatic assays

Shikimate dehydrogenase (SHD, EC 1.1.1.25) was extracted as described by Díaz and Merino [4]. Fresh roots (0.5 g) were ground at 4 °C in 0.1 M potassium phosphate buffer (pH 7.4), 2 mM ethylene diamine tetraacetic acid (EDTA), 8 mM β-mercaptoethanol, and 0.05 g of polyvinylpyrrolidone (PVP). Homogenates were centrifuged (2200*g*, 10 min) and the supernatant was used as the enzyme preparation. Enzyme activity was determined with shikimate as substrate by quantifying the production of NADPH spectrophotometrically at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$). The reaction mixture (4 mM shikimic acid in 0.1 M Tris–HCl buffer, pH 9.0, and a suitable amount of enzyme extract in a final volume of 900 μL) was incubated at room temperature. The reaction was started by adding 100 μL of 20 mM NADP, and NADP reduction was monitored for 3 min. The results were expressed as nmol NADPH $min^{-1}\ g^{-1}$ fresh weight.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was extracted as described by Ferrarese et al. [14]. Fresh roots (2 g) were ground at 4 °C in 0.1 M sodium borate buffer (pH 8.8). Homogenates were centrifuged (2200*g*, 15 min) and the supernatant was used as the enzyme preparation. The reaction mixture (100 μmol sodium borate buffer pH 8.7 and a suitable amount of enzyme extract in a final volume of 1.5 mL) was incubated at 40 °C for 5 min for the PAL activity assay. Fifteen micromoles of L-phenylalanine were added to start the reaction, which was stopped after 1 h of incubation by the addition of 50 μL of 5 N HCl. Samples were filtered through a 0.45 μm disposable syringe filter and analyzed (20 μL) with a Shimadzu® Liquid Chromatograph (Tokyo, Japan) equipped with a LC-10AD pump, a Rheodyne® injector, a SPD-10A UV detector, a CBM-101 Communications Bus Module and a Class-CR10 workstation system. A reversed-phase Shimpack® CLC-ODS (M) column (150 × 4.6 mm, 5 μm) was used at 30 °C, with an equivalent pre-column (10 × 4.6 mm). The mobile phase was methanol:water (70:30) with a flow rate of 0.5 mL min^{-1}. Absorption was measured at 275 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). *t*-Cinnamate, the product of PAL, was identified by comparing its retention time with standard values. Parallel controls without L-phenylalanine or with *t*-cinnamate (added as an internal standard in the reaction mixture) were performed as described elsewhere [14]. PAL activity was expressed as μmol *t*-cinnamate $h^{-1}\ g^{-1}$ fresh weight.

2.3. Shikimate quantification

Fresh roots (0.5 g) were ground in 1.5 mL of 0.25 N HCl. Homogenates were centrifuged (4 °C, 1200*g*, 10 min) and the supernatant was used to determine the shikimate content. Samples were filtered through a 0.45 μm disposable syringe filter and analyzed (20 μL) with a high performance liquid chromatography (HPLC) system (Shimadzu®, Tokyo, Japan), as described above. The mobile phase was 3.5 mM phosphoric acid with a flow rate of 0.8 mL min^{-1}. Shikimate was identified at 220 nm by comparing its retention time with a standard value [7]. Parallel controls with shikimate added as an internal standard in the reaction mixture were performed. Results were expressed as μg shikimate g^{-1} fresh weight.

2.4. Phenolic compound quantification

Hydroxybenzoic (protocatechuic, *p*-hydroxybenzoic, and vanillic) and hydroxycinnamic (ferulic, *p*-coumaric, caffeic, and sinapic) acids were determined by HPLC (Shimadzu®, Tokyo, Japan). Dry root (0.25 g) was boiled for 30 min in 5 mL of 2 N HCl. After cooling, the homogenate was filtered through a 0.45 μm disposable syringe filter and analyzed (20 μL) chromatographically, as described above. The mobile phase was methanol:acetic acid (30:4) with a flow rate of 0.8 mL min^{-1}. The compounds were identified at 254 nm by comparing their retention times with standard values [15]. Parallel controls with hydroxybenzoic and hydroxycinnamic acids added as internal standard in the reaction mixture were performed. Results were expressed as mg g^{-1} dry weight.

2.5. Lignin quantification

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 [16]. 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); 3× with 1% (v/v) Triton® X-100 in pH 7.0 buffer (7 mL); 2× with 1 M NaCl in pH 7.0 buffer (7 mL); 2× with distilled