A simple chromatographic assay to discriminate between glyphosate-resistant and susceptible soybean (Glycine max) cultivars

E.A. Bonini, M.L.L. Ferrarese, R. Marchiosi, P.C. Zonetti, O. Ferrarese-Filho *

1. Introduction

With the increasing development of genetically modified plants, new regulations for the manipulation, growth and use of these organisms are being implemented in several countries. These regulations demand reliable methods for detecting small differences between conventional and genetically modified plant species, processed/unprocessed foods, flavor, meal, grain and seeds. In general, such methods include nucleic acid amplification methods, protein-based methods and detection of enzymatic activities, among others (Deisingh and Badrie, 2005).

The shikimate pathway, one of the major biosynthetic pathways in higher plants, has been an attractive target for the design of herbicidal agents. Its penultimate step results in the reversible formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate from shikimate-3-phosphate and phosphoenolpyruvate. The reaction is catalyzed by EPSP synthase (EC 2.5.1.19), which is the only cellular target for the herbicide glyphosate [(N-phosphonomethyl)glycine] inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, causing a massive accumulation of the metabolite shikimate. This phenomenon does not occur in glyphosate-resistant soybean due to the presence of a gene encoding glyphosate-insensitive EPSP synthase. This study proposes a simple and reliable assay as an alternative tool for differentiating glyphosate-resistant from susceptible soybean cultivars. The assay is based on a single extraction of leaf or root tissue. Shikimate is quantified by reversed-phase high-performance liquid chromatography at 220 nm and isocratic elution with phosphoric acid. After glyphosate treatment, tissues of glyphosate-susceptible plants were found to massively accumulate shikimate, whereas the tissues of glyphosate-resistant plants did not accumulate the metabolite.

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Abstract

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exposed soybean tissues and to apply this assay for distinguishing between glyphosate-resistant and susceptible soybean cultivars.

2. Materials and methods

2.1. Experimental procedures

To discriminate glyphosate-resistant from glyphosate-susceptible soybean cultivars, three protocols were followed: (1) with roots of seedlings grown after three days of seed germination, (2) with roots of seedlings grown after three days of germination followed by incubation in nutrient solution for 24 h (hydroponic assay), and (3) with leaves of plants grown under greenhouse conditions.

For the germination procedure, soybean [Glycine max L. Merr. cv. BRSS-133 – glyphosate-susceptible – and cv. BRSS-245RR – glyphosate-resistant – (Embrapa Soja, Brazil)] seeds (200 each) were immersed either in 300 mL of 0.1, 0.2 or 1 mM glyphosate solution or in water (control) for 4 h (Marchiosi et al., 2009). After this short immersion period, the seeds were dark-germinated (at 25 °C) on three sheets of filter paper moistened with water. Three days later, the roots were excised, and the fresh tissues were immediately used for shikimate quantification.

For the hydroponic procedure, seeds were dark-germinated (at 25 °C) on three sheets of moistened filter paper (Soares et al., 2007). Twenty-five 3-day-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred into a glass container (10 × 16 cm) filled with 200 mL of half-strength Hoagland's solution (pH 6.0) with or without 1, 1.5 or 2 mM glyphosate. The containers were kept in a growth chamber (25 °C, 12-h photoperiod, irradiance of 280 μmol m⁻² s⁻¹). The nutrient solution was aerated continuously by air bubbling. Roots were excised from seedlings after 24 h, and the fresh tissues were immediately used for shikimate quantification.

For the greenhouse procedure, seeds were planted in 5 L-plastic pots containing a mixture of sandy soil (920 g kg⁻¹ sand and 30 g kg⁻¹ clay, pH 6.2). Ten seeds were sown (at 1 cm of depth) in each of the pots, which were watered daily. The greenhouse was maintained at 24 °C (±3 °C) with natural light. After one week, plants were thinned to four uniform plants per pot. When the plants were three weeks old (V3 stage, one trifoliate leaf), each pot was uniformly sprayed by using a hand-held sprayer containing 15 mL of a solution of glyphosate (2, 10 or 15 mM) corresponding to 0.051, 0.254 or 0.383 kg ae ha⁻¹, respectively. Application was made to provide more complete foliar coverage. For each cultivar, a similar control was sprayed with water. Three days after treatment, leaves of the second trifoliate were harvested and the fresh tissues were immediately used for shikimate quantification.

Glyphosate (N-(phosphonomethyl)glycine), 96% purity, and shikimic acid, min. 99% purity, were purchased from Sigma (St. Louis, MO), and all other reagents used were either of chromatographic grade or the purest grade available.

2.2. Shikimate quantification

Fresh tissues (0.5 g) were ground in 1.5 mL of 0.25N HCl. Homogenates were centrifuged (1200 × g, 10 min) at room temperature and the supernatant was used to measure the shikimate content (Singh and Shaner, 1998). Samples were appropriately diluted and filtered through a 0.45 μm disposable syringe filter (Hamilton® Co., Nevada, USA) prior to chromatographic analyses. Samples (20 μL) were analyzed 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 room temperature, together with the same type of pre-column (10 × 4.6 mm). The mobile phase was 3.5 mM phosphoric acid with a flow rate of 0.8 mL min⁻¹ for an isocratic run of 10 min (Lydon and Duke, 1988). Absorption was measured at 220 nm, a wavelength that was previously determined by spectrophotometric scanning of a shikimic acid authentic standard dissolved in the mobile phase. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). Shikimate was identified by comparing its retention time with that of a 20 μM standard. Parallel controls with shikimate added as an internal standard in the reaction mixture were performed. The shikimate concentration in the sample was equal to the standard concentration × the peak area of the sample/peak area of the standard. A six-point calibration curve with shikimic acid concentrations ranging from 1.75 to 17.5 μg mL⁻¹ was used to externally quantify shikimate levels in the tissue extracts. Results were expressed as mg shikimate g⁻¹ fresh weight, representing the means of four independent experiments ± S.E. The recovery for the extraction method was determined by using three root samples (0.5 g, control) spiked with 20 μM shikimic acid before extraction. The recovery obtained was 94.8 (±4.5)%. For proposed method, the limits of detection (LOD) and quantification (LOQ) for shikimic acid were determined as three and ten times base line noise, respectively (Ribani et al., 2007). The LOD and LOQ were 0.02 and 0.07 μg mL⁻¹, respectively; these low values are indicative of the high sensitivity of the method.

2.3. Statistical analysis

Significant differences were verified by one-way variance analysis with the Sisvar package (Version 4.6, UFLA, Brazil). Differences between means were tested according to the Scott–Knott test, and comparisons were performed at 5% significance.

3. Results and discussion

It is well known that the herbicide glyphosate inhibits EPSP synthase, resulting in the accumulation of shikimate, the dephos-
phorylated substrate of the enzyme. As a result of this inhibition, a massive accumulation of shikimate occurs, and this phenomenon has been used to verify injury caused by glyphosate on plant tissues (Pline et al., 2002; Shaner et al., 2005; Buehring et al., 2007).

In the present study, shikimate was assayed chromatographically in soybean tissues for differentiating glyphosate-resistant from glyphosate-susceptible cultivars. Fig. 1A shows a representative chromatographic profile of the shikimate standard, which eluted at 5.40 min. The figure also shows the profile of shikimate (retention time = 5.36 min) in leaf extracts of susceptible soybean, after 1 mM-herbicide exposure (Fig. 1B), indicating an accumulation of this metabolite. The HPLC-profile also demonstrates that shikimate is undetectable in tissues of glyphosate-resistant soybean (Fig. 1C) since its measurements do not present sufficient confidence for quantification. This confirms the herbicide resistance of this transgenic cultivar (Padgette et al., 1995). These patterns were similar for all samples analyzed in this work.

To validate the proposed assay, three different protocols were used, as described in Section 2. Shikimate levels were measured in root or leaf tissue of glyphosate-susceptible and glyphosate-resistant soybean in seed germination (Fig. 2A), hydroponic (Fig. 2B) and greenhouse (Fig. 2C) conditions. Under the first experimental condition, roots of susceptible soybean seedlings accumulated 0.3 to 0.5 mg shikimate g⁻¹ fresh weight after 0.1 to 1 mM exposure (Fig. 2A). At 2 and 5 mM, glyphosate also led to the accumulation of shikimate in BRS-133 soybean roots, reinforcing the susceptibility of this cultivar to the herbicide (Marchiosi et al., 2009). Under the second condition, roots of susceptible soybean accumulated 0.6 to 1.7 mg shikimate g⁻¹ fresh weight after 1 to 2 mM glyphosate treatment (Fig. 2B). No significant change was detected in roots below 1 mM herbicide exposure (not shown). As a consequence of herbicide action, leaves of susceptible soybean accumulated 1.6 and 6.8 mg shikimate g⁻¹ fresh weight after 10 and 15 mM glyphosate treatment, respectively (Fig. 2C). No significant change was detected in leaves below 2 mM herbicide exposure, indicating that this concentration was not sufficient to induce shikimate accumulation. In glyphosate-treated susceptible soybean, similar trends have been reported for apical meristem (Singh and Shaner, 1998) and leaves (Shaner et al., 2005; Nandula et al., 2007). In these reports, shikimate accumulation was found to be proportional to glyphosate application rates in the tissue samples tested. Consistent with the above-mentioned reports, glyphosate application in the current study resulted in shikimate accumulation in susceptible (BRS-133) soybean tissues and no accumulation of shikimate in tissue from glyphosate-resistant (BRS-245RR) soybean (Fig. 2A–C).

In summary, the data presented in this study indicate that our methodology is highly sensitive and also that it may be applied satisfactorily to distinguish glyphosate-resistant from glyphosate-susceptible soybean cultivars.

4. Conclusion

The isocratic HPLC assay described herein is simple, quick and reliable for the quantification of shikimate accumulated in tissues of susceptible soybean exposed to glyphosate. No clean-up procedure of the crude extract is needed prior to analysis. Significant differences were found between the cultivars assayed for all experimental conditions applied here, indicating that this procedure may represent a useful alternative tool for distinguishing between glyphosate-resistant and glyphosate-susceptible soybean cultivars. All experimental protocols may be applied for this purpose, but seed germination procedure is firmly recommended due to its simplicity, speed and sensitivity.

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