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Glyphosate accumulation, translocation, and biological effects in *Coffea arabica* after single and multiple exposures

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ABSTRACT

In perennial crops like coffee, glyphosate drift exposure can occur multiple times during its commercial life span. Due to limited glyphosate degradation in higher plants, a potential accumulation of glyphosate could lead to increased biological effects with increased exposure frequency. In this study, we investigated glyphosate translocation over time, and its concentration and biological effects after single and multiple simulated spray-drift exposures. Additionally, shikimic acid/glyphosate ratios were used as biomarkers for glyphosate binding to its target enzyme.

Four weeks after the exposure, glyphosate was continuously translocated. Shikimic acid levels were linear correlated with glyphosate levels. After two months, however, glyphosate appeared to have reduced activity. In the greenhouse, multiple applications resulted in higher internal glyphosate concentrations. The time of application, however, was more important regarding biological effects than the number of applications both in the greenhouse and in the field. In the field, berry yield, the most important biological response variable, was reduced 26% by the first out of four sequential applications of glyphosate at 64 g a.e. ha^{-1} each. The three subsequent applications did not reduce yield any further.

1. Introduction

The non-selective, post-emergence herbicide glyphosate [N-(phosphonomethyl) glycine], was first commercialized approximately 40 years ago. Today, it is the most used plant protection chemical worldwide (Steinmann et al., 2012). Once taken up, glyphosate is mobile in both xylem and phloem. Because of fast reloading into the phloem (Preston and Wakelin, 2008), glyphosate translocation is, however, predominant in the phloem from source to sink (Shaner, 2009), following the path of sucrose and other photosynthates. Its target site is the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and upon its inhibition by glyphosate shikimic acid accumulates. The mode of action and the resulting accumulation of shikimic acid are well documented (Amrhein et al., 1980; De Maria et al., 2006; Schonbrunn et al., 2001). An increased level of shikimic acid can be used as biomarker for a recent exposure to glyphosate. An accumulation of shikimic acid can additionally be used as an effect measure on a molecular level indicating the degree of EPSPS inhibition.

Because of its non-selective mode of action, glyphosate possesses the risk of damaging the crop through unintended spray drift. Various studies have been carried out to evaluate glyphosate crop toxicity via spray drift simulation experiments. Annual crops such as corn (Buehring et al., 2007), rice (Koger et al., 2005), soybean (Ding et al., 2011), peanut (Lassiter et al., 2007), and wheat (Rolder et al., 2007) were investigated as well as perennial plants grown as annuals including potato (Felix et al., 2011) and tomato (Gilreath et al., 2001), and woody perennial plants such as grape vine (Al-Khatib et al., 1993), cherry trees (Al-Khatib et al., 1992) and coffee (Franca et al., 2013; Schrübbers et al., 2014). The latter group, in contrast to annual plants and perennials grown as annuals, is potentially exposed many times during their commercial lifespan.

Coffee is grown in regions without cold winters; making weed control necessary all year round; especially in the rainy season with favorable conditions for weed growth (Staver et al., 2001). Multiple exposures make an evaluation of glyphosate accumulation important for perennial crops, as a potential accumulation could lead to increased effects with time. Glyphosate already present in the plant could add to an otherwise non-damaging dose to reach an internal concentration high enough to cause a toxic effect. Damage will only take place if glyphosate is not sufficiently degraded, excreted, and/or stored in a place or form that avoids its interaction with

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the target enzyme within the interval of the multiple applications (Dayan et al., 2010; Duke, 2011; Pfleeger et al., 2014).

Despite a reported slow or absent glyphosate degradation in higher plants (Duke, 2011), the literature is sparse on studies evaluating glyphosate concentration and effects in plants several weeks after single and, especially, multiple exposures (Pfleeger et al., 2014). An example are glyphosate resistant (GR) soybean leaves and stems, in which 3–4 mg glyphosate kg^{-1} were detected several weeks after single or multiple applications (Arregui et al., 2004). Generally, higher glyphosate concentrations were found in plants exposed multiple times to the herbicide.

Information on the risk of accumulating internal concentrations by multiple exposures could potentially also be obtained from degradation curves after a single application, because an accumulation of glyphosate requires the chemical to be still present from the previous application. However, a decrease in glyphosate concentration might mainly be caused by a dilution effect due to plant growth, as shown in corn (Bernal et al., 2012). This dilution effect can be difficult to estimate for longer periods when plant growth varies with plant age and environmental conditions. Additionally, the biological effect of glyphosate will depend on the mobility of the herbicide within the plant both between organs and cell compartments, as the target site EPSPS is mainly located in the cytoplasm of active meristems (Shaner, 2009). Studies with GR horseweed (Ge et al., 2010, 2011) have shown that translocation of glyphosate to vacuoles can decrease the biological effect and thereby act as a resistance mechanism. As aforesaid, the accumulation of shikimic acid is an important identification criterion for glyphosate reaching its target enzyme. The correlation of glyphosate and shikimic acid concentrations over time has to our knowledge not been evaluated. The few studies we are aware of analyzing both analytes in the same sample either used GR soybean plants (Duke et al., 2003; Reddy et al., 2004) that do not accumulate shikimic acid after glyphosate exposure; or investigated only short intervals after application, i.e. 72 h in cotton (Pline et al., 2002) or 7 days using several plant species (Reddy et al., 2008). The limited glyphosate degradation in higher plants (Duke, 2011), however, makes the evaluation of the shikimic acid/glyphosate ratio over longer intervals interesting.

When glyphosate is degraded, its main metabolite is aminomethylphosphonic acid (AMPA). AMPA is considered phytotoxic in itself; but less potent than glyphosate and has a different mode of action, as no shikimic acid accumulation is observed in plants exposed to AMPA only (Reddy et al., 2004). To investigate the biological effects of glyphosate in a plant over time, knowledge of AMPA concentrations are therefore necessary.

The aim of this study was to investigate glyphosate accumulation, translocation and biological effects of one and multiple sub lethal doses in coffee plants over extended periods. Coffee was chosen as an example of a perennial crop typically experiencing several glyphosate applications per year. It was also selected for its high economic importance and because glyphosate related damage symptoms are often observed in coffee plantations despite careful glyphosate application (Arizaleta et al., 2008; Franca et al., 2010; Rodrigues et al., 2003). Coffee leaves were analyzed for glyphosate and AMPA after a single glyphosate application in different plant parts (compartments) at different intervals to obtain information on glyphosate translocation in the plant and thereby its ability to reach the target site after several weeks. Additionally, samples previously analyzed in our laboratory (Schrübbers et al., 2014) for shikimic acid were analyzed for glyphosate and correlated to evaluate in situ target site binding. Multiple exposures were applied in a greenhouse and field trial to estimate possible glyphosate accumulation and the effect on growth, plant health and coffee yield. Because of the slow degradation in higher plants, we hypothesize higher glyphosate concentration and increased phytotoxicity would be observed after several glyphosate applications.

2. Materials and methods

2.1. Chemicals and solutions

Glyphosate (purity ≥97.0%) and AMPA (99%) were purchased from Sigma–Aldrich (Steinheim, Germany). The isotope labeled internal standards 1,2-$^{13}C2$ ^{15}N glyphosate (98%) and ^{13}C ^{15}N AMPA (99% for ^{13}C, 34% for ^{15}N) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The organic solvents methanol and acetonitrile were HPLC grade, obtained from Rathburn (Walkerburn, Scotland). For the mobile phase, LC–MS grade (Chromasolv®) acetonitrile from Sigma–Aldrich (Steinheim, Germany) was used. Dichloromethane, potassium hydroxide (p.a.) and boric acid (all EMSURE® p.a.) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (37%, AnalaR NORMAPUR) and sodium hydroxide were obtained from VWR (Fontenay-sous-Bois, France), and J.T. Baker (Deventer, Netherlands), respectively. Ammonium formate (MS grade ≥99.0%), ammonium hydroxide (LC–MS grade), formic acid (98%), and 9-fluorenylmethylchloroformate (FMOC chloride; ≥99%) were all from Sigma–Aldrich (Steinheim, Germany).

All FMOC solutions were prepared the same day in acetonitrile. Borate buffer solutions consisted of 500 mM boric acid adjusted to pH 9 with 1 M NaOH. Stock solutions were prepared by dissolving 25.0 mg powder of the analyte in 25.0 mL MilliQ water. To dissolve the material, the solutions were sonicated with a Branson 1510E-DTH (Branson Ultrasonic Corporation, USA) at 40 °C; 2 × 8 min for glyphosate and 1 × 8 min for AMPA. The internal standards were obtained as aqueous solutions from the manufacturer. Dilutions to prepare working solution were made in MilliQ. Standard curves were prepared by mixing different volumes of working solutions and water to give 1.2 mL with 1.2 mL borate buffer in 15 mL falcon tubes. Subsequently, 600 μL 10 mM FMOC solution were added, mixed and let stand for one hour. After the derivatization was completed, 2 mL dichloromethane were added, vortex mixed, centrifuged at 4000 rpm and the upper water phase was filtered (0.2 μm PTFE membrane, 13 mm syringe filer, VWR International, USA) into a LC-vial prior to injection.

2.2. Sample preparation

Glyphosate was determined according to a method previously developed for coffee leaves (Schrübbers et al., 2015). The method was carried out with minor alterations and an instrumental change; the high performance liquid chromatography (HPLC) single quadrupole of the original method was substituted by an ultra performance liquid chromatography (UPLC) triple quadrupole (QqQ) tandem mass spectrometer system. Briefly coffee leaves (*Coffea arabica* L.) were stored for at least one night at −80 °C and subsequently freeze-dried (Hetosicc, Heto, Birkerød Denmark). To homogenize and to reduce the particle size the sample was placed in a 250 mL PEHD bottle together with three stainless steel balls (15 mm in diameter) and ground to a fine powder by shaking using a conventional paint mixer (SO-40a, Fluid Management Europe, IDEX Corporation, Sassenheim, Netherlands). Samples for the correlation of shikimic acid and glyphosate concentration were prepared as described in Schrübbers et al. (2014) and 0.5 g was used for the analysis of glyphosate. For all other samples 1 g or all available material was used. The powder was added to a 50 mL falcon tube and spiked with internal standard (0.8 μg and 0.38 μg 1,2-$^{13}C_2$ ^{15}N glyphosate and ^{13}C ^{15}N AMPA, respectively). The extraction was carried out with water (18 mL) and 1 M HCl (2 mL). The sample was shaken, sonicated (Branson 1200, Branson Ultrasonic Corporation, USA) for 5 min and thereafter placed on a horizontal shaker (build in-house) for 30 min at 1.1 Hz. After the extraction, the sample was centrifuged for 5 min at 4000 rpm at 21 °C. An aliquot of the supernatant (10 mL) was loaded to a 6 mL, 500 mg Strata-X solid