SHIKIMIC ACID EXTRACTION FROM LEAF TISSUE OF SOURGRASS (*Digitaria insularis* L.)

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SUMMARY

The microwave-assisted extraction (MWAE) method using leaf tissue is simple and efficient to measure endogenous shikimic acid accumulation in plants. The MWAE method has mostly used green leaf tissue collected from plants up to 7 days after glyphosate treatment. We investigated if brown leaf tissue from plants at 21 days after being treated with glyphosate could be used as source material to extract shikimic acid by the MWAE method. Two populations of sourgrass (*Digitaria insularis* L.) were sprayed with increasing glyphosate rates (0; 270; 540; 1,080; 2,160 and 4,320 g a.e. ha^{-1}). At 21 days after treatment, shikimic acid was extracted by the MWAE method and quantified by high performance liquid chromatography (HPLC). Endogenous shikimic acid was successfully extracted and quantified in all treatments. The viability of extracting shikimic acid after plant metabolism is inactive may help in issues related to glyphosate-induced damage in sensitive plants.
Key words: plant tissue, shikimic acid accumulation, glyphosate.

RESUMEN

El método de extracción asistida por microondas (MEAM) utilizando tejido de hoja es simple y eficiente para medir la acumulación de ácido shikímico endógeno en plantas. El método MEAM ha utilizado sobretodo tejido de hoja verde obtenido de plantas de hasta 7 días después de tratamiento con glifosato. Hemos investigado si la hoja de tejido marrón de las plantas a los 21 días después de haber sido tratadas con glifosato podría ser utilizada como material de origen para extraer el ácido shikímico por el método MEAM. Dos poblaciones de camalote (*Digitaria insularis* L.) fueron aplicadas con tasas crecientes de glifosato (0; 270; 540; 1080; 2160 y 4320 g e.a. ha^{-1}). A los 21 días después del tratamiento, el ácido shikímico fue extraído por el método MWAE y cuantificado por cromatografía líquida de alta resolución (HPLC). Ácido shikímico endógeno fue extraído y cuantificado con éxito en todos los tratamientos. La viabilidad de la extracción de ácido shikímico después que el metabolismo de la planta está inactivo puede ayudar en temas relacionados con el daño inducido por el glifosato en plantas sensibles.
Palabras clave: tejido de planta, acumulación de ácido shikímico, glifosato.

INTRODUCTION

Shikimic acid is a precursor of primary metabolites, including aromatic amino acids and other compounds possessing the benzene ring. Shikimic acid is also a precursor of oseltamivir, which has been considered the most effective compound against avian and swine flus [1]. In herbicide research, shikimic acid measurement became important as a technique not only to quantify damage induced by herbicides in susceptible plants [2,3], but also to rapidly detect glyphosate resistance in weeds [4]. As soon as glyphosate-resistant crops were launched, weed control started to rely mostly on glyphosate, leading, some years later, to the selection of glyphosate-resistant weeds [5]. Sourgrass (*Digitaria insularis* L.) biotypes resistant to glyphosate have been a management issue in Brazilian

agriculture. This weed species is perennial and produce rhizomes after its establishment, occurring during the entire year in Brazilian weather conditions [6]. A microwave-assisted extraction (MWAE) for determining shikimic acid accumulation in plants was developed [7], in which smaller amounts of matrix and solvents are used, compared to the existing methods. Thus far, the MWAE method has used material collected from plants whose metabolisms were still present, at 7 or 10 days after glyphosate treatment. Therefore, we investigated if brown leaf tissue from sourgrass plants, at 21 days after glyphosate treatment, could be used for shikimic acid extraction by the MWAE method.

MATERIAL AND METHODS

Plant Material. Seeds of a sourgrass population with suspected resistance to glyphosate were collected from a commercial coffee plantation located in Espírito Santo State, Brazil. A known susceptible sourgrass population was included as a control.

Whole-Plant Dose-Response to Glyphosate. When plants of the susceptible (S1) and the suspected resistant (S2) sourgrass populations reached the three- to four-leaf stage, glyphosate (Roundup WG) was applied at six rates: 0 (control), 0.25X, 0.5X, 1X, 2X and 4X, where X corresponds to the glyphosate recommended field rate for sourgrass control (1.5 kg of commercial product per ha). Treatments were applied with a CO_2 backpack sprayer at a flow rate of 150 $L.ha^{-1}$. Visual control was recorded at 21 days after treatment (DAT) based on the scale developed by the Latin American Weed Association (ALAM)[8], where 0% indicates absence of control and 100% indicates absolute control. Aboveground biomass per pot was harvested into paper bags and was kept in an airflow chamber at 65ºC for 48 hours. Treatments (factorial combination of glyphosate rates and sourgrass populations) were completely randomized and replicated four times.

Microwave-assisted Shikimic Acid Extraction. We followed the method developed by Matallo et al. (2009). Whole leaves from the dry aboveground biomass were ground. The material was pooled and 400mg were placed into plastic tubes. Ten milliliters of acidified distilled water (pH=2.0) was added to each sample. The mix was shaken for 5s and microwaved for 10s. Extracts were filtered and transferred into chromatographic vials.

HPLC Quantification. A Shimadzu LC 2010 chromatograph was used. Detection of shikimic acid was performed at a wavelength of 212 nm. Twenty µL of sample was injected per column. The isocratic system used deionized water (pH=3.0): methanol (95:5) and a flow rate of 1.0 $mL.min^{-1}$. The total running time was 10 min. Concentration values were converted to shikimic acid/dry weight (w/w) based on a standard curve using the control plants and known concentrations of shikimic acid ranging from 2.04 to 407.2 $\mu g.mL^{-1}$.

Data Analysis. Visual control and shikimic acid accumulation data were analyzed using a nonlinear regression model [9]. Dose-response models were constructed using a four-parameter log-logistic equation: $y = c + \{d - c / 1 + \exp[b^*(\log x - \log e)]\}$, where y is visual control as a percent of the untreated control or shikimic acid accumulation, e (ED_{50}) is the glyphosate rate to provide 50% of visual control based on the untreated control or 50% of shikimic acid accumulation, x is the glyphosate rate, and b is the slope at e. The lower and upper limits of the curve are represented by c and d, respectively.

RESULTS AND DISCUSSION

Whole-Plant Dose-Response Experiment. The dose-response experiment and shikimic acid accumulation assays confirmed glyphosate susceptibility in the known susceptible population S1 and revealed that the suspected resistant population S2 was not glyphosate resistant. Plants from the two populations were both affected by glyphosate. Values for ED_{50} differed between populations (Tables 1 and 2). Despite that, at the recommended field rate for sourgrass control in Brazil [10], visual control of S1 and S2 plants was nearly 100% (Figures 1 and 3). Shikimic acid accumulated as glyphosate rates increased (Figure 2). Consistent with the visual control data, values for ED_{50} were significantly different between S1 and S2 for shikimic acid accumulation (Table 2). For the two populations, glyphosate rates required to provide 50% of visual control or 50% of shikimic acid accumulation were below the recommended field rate in kilograms of commercial product per hectare (Tables 1 and 2).

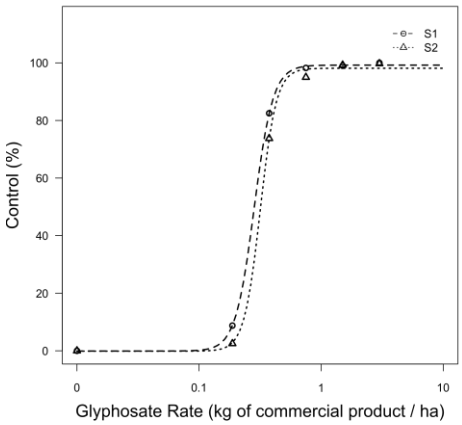


Figure 1. Visual control of S1 and S2 populations at 21 DAT with glyphosate.

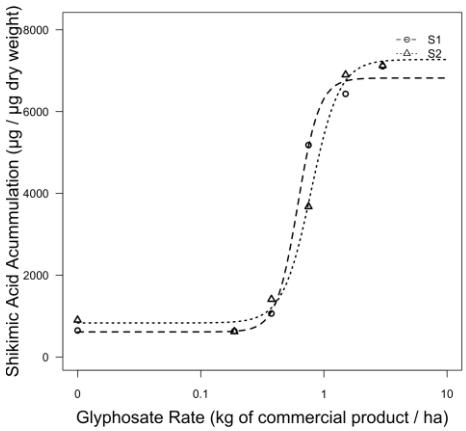


Figure 2. Shikimic acid accumulation in leaves from S1 and S2 plants, 21 DAT with glyphosate.

Table 1. Parameter estimates for the log-logistic regression model used in the visual control data. Parameter abbreviations: *d*, upper limit; *b*, slope at ED_{50}; ED_{50}, glyphosate rate that results in 50% of visual control compared to the untreated control.

Population	Parameter	Estimate	S.E.	95% C.I.	P-value
S1	*b*	-5.65	0.55	(-6.76; -4.53)	< 0.0001
	c	-0.09	2.13	(-4.40; 4.22)	0.9665
	d	99.22	1.25	(96.68; 100)	< 0.0001
	ED_{50}	0.28	0.009	(0.26; 0.30)	< 0.0001
S2	*b*	-6.60	1.48	(-9.61; -3.59)	0.0001
	c	-0.12	2.12	(-4.41; 4.16)	0.9534
	d	98.13	1.25	(95.60; 100)	< 0.0001
	ED_{50}	0.31	0.01	(0.29; 0.34)	< 0.0001

[a] Abbreviation: S.E., standard error, C.I., confidence interval

Table 2. Parameter estimates for the log-logistic regression model used in the shikimic acid accumulation data. Parameter abbreviations: *d*, upper limit; *b*, slope at ED_{50}; ED_{50}, glyphosate rate that provides 50% shikimic acid accumulation, based on the untreated control.

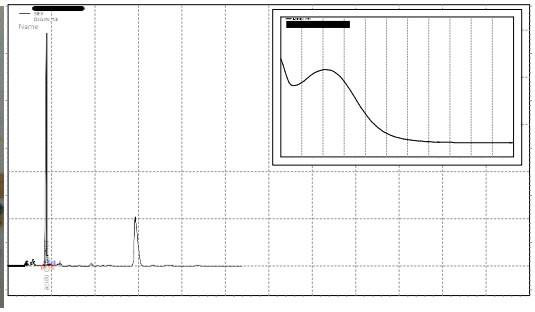
Population	Parameter	Estimate	S.E.	95% C.I.	P-value
S1	*b*	-4.95	0.84	(-6.68; -3.23)	< 0.0001
	c	612.72	147.12	(311.35; 914.08)	0.0003
	d	6821.06	153.45	(6506.72; 7135.39)	< 0.0001
	ED_{50}	0.61	0.02	(0.56; 0.66)	< 0.0001
S2	*b*	-3.78	0.69	(-5.21; -2.34)	< 0.0001
	c	832.08	144.65	(535.78; 1128.39)	< 0.0001
	d	7273.76	204.92	(6854.01; 7693.52)	< 0.0001
	ED_{50}	0.78	0.03	(0.72; 0.85)	< 0.0001

[a] Abbreviation: S.E., standard error, C.I., confidence interval

High Performance Liquid Chromatography (HPLC) Analyses: Chromatograms provided by the HPLC analyses showed that the gradual elution provided good separation of shikimic acid with no interference in all treatments. The shikimic acid absorbance spectrum is shown in the insets of chromatograms (Figure 4). Most methods for measuring shikimic acid accumulation require fresh tissue, homogenized or ground [11]. Brown and dried leaves from sourgrass plants killed with glyphosate (Figure 3) showed to be as good as dried leaves from the control plants for using in the MWAE method. The entire shikimate pathway represents one of the constituents of the primary cell metabolism. While 5-enolpyruvylshikimate-3- phosphate (EPSP) synthase is blocked by glyphosate, shikimic acid accumulates in the leaf tissue [12]. At 21 DAT, sourgrass plants treated with glyphosate, at the recommended rate on, were killed, that

is, the metabolic processes of the treated plants had been stopped at this time. After plant death at 15 DAT, accumulated shikimic acid remained in the leaf tissue and did not degrade in the following 6 days. Thus, sourgrass leaf tissue from susceptible plants does not need to be metabolically active before harvest for using in the MWAE method. Shikimic acid can be extracted from dead sourgrass plants, at 21 DAT with glyphosate.

Figure 3. Illustration of S2 sourgrass plants: control plants (above) and plants killed by the recommended glyphosate rate, 21 DAT (below).



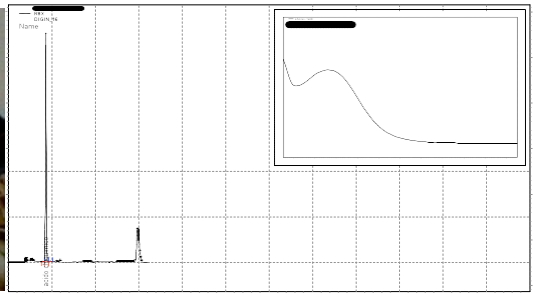


Figure 4. Illustration of chromatograms at 212 nm of shikimic acid in S1 (above) and S2 (below) samples treated at glyphosate rate 8X, 21 DAT. Inset: absorption spectrum of shikimic acid.

CONCLUSIONS

Our results showed that: 1) shikimic acid can be extracted and quantified using leaves from sourgrass plants killed with glyphosate, 2) sourgrass plants treated with glyphosate can hold 21 days to be used in the MWAE method, 3) further studies can be conducted to test other plant species, and 4) late shikimic acid determinations may be valuable in issues related to glyphosate utilization in susceptible plants.

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